

**THE MICROBIAL DEGRADATION  
OF WOOL IN  
THE MARINE ENVIRONMENT.**

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## ABSTRACT.

The Wool Research Organisation of New Zealand has developed Woolspill<sup>TM</sup>, a sorbent for the removal of oil from water. Woolspill<sup>TM</sup> employs small balls of wool known as knops which are capable of adsorbing up to forty times their own weight in oil. It is envisioned that this product will be used for the clean-up of oil spills in the marine environment. As the fate of wool in the marine environment is unknown, an investigation into its microbial degradation was undertaken.

Flask cultures of wool in sea water were established in the laboratory, and biochemical indicators of wool degradation monitored. In cultures containing microbes enriched from sea water, wool was degraded at a much slower rate, than by pure cultures of the keratinolytic bacteria *Streptomyces fradiae* and *Lysobacter*. Although wool degradation in sea water was observed by light microscopy, no soluble sulfhydryls were detected, possibly indicating that the keratin component of the wool fibre (and most resistant to microbial degradation) was not being degraded by the microbial populations present in sea water. The adsorption of oil upon wool was found to reduce wool degradation in the cultures of mixed marine microbes, and cultures of the keratinolytic bacteria *Lysobacter*, and *S. fradiae*.

Microbes were enriched on wool oil booms placed *in situ* in Lyttleton Harbour. At monthly intervals samples were removed from the booms for the isolation of microbes, and microscopic studies. Microbes were screened for their wool degrading ability, through the use of a plate assay containing soluble keratin derived from poultry feathers. Bacteria, not fungi were observed to play an important role in the degradation of wool in the marine environment. Two wool degrading bacteria were isolated. They were tentatively placed in the genera *Alteromonas*, and *Oceanospirillum*.

These results demonstrated that wool was degraded in the marine environment.

## ABBREVIATIONS.

ASW	Artificial Sea Water
ATCC	American Type Culture Collection
DMSO	Dimethyl Sulfoxide
DTNB	5,5'-Dithiobis-(2-nitrobenzoic Acid)
EDTA	Ethylenediaminetetraacetic Acid
GMA	Gelatin Marine Agar
KS	Soluble Keratin
LBM	<i>Lysobacter</i> Basal Medium
M2	Marine Actinomyetes Agar
MA	Marine Agar 2216
MB	Marine Broth 2216
MFA	Marine Fungi Agar
MFGA	Marine Fungi Gelatin Agar
MKA	Marine Keratin Agar
MPRBA	Marine Phenol Red Base Agar
NCIMB	National Culture of Industrial and Marine Bacteria
LPC	<i>Lysobacter</i> Plate Culture Agar
PAB	Phenol Aniline Blue
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Agar
PEP	<i>Lysobacter</i> Culture Broth
SEM	Scanning Electron Microscopy
SFBA	<i>Streptomyces fradiae</i> Basal Agar
SFBM	<i>Streptomyces fradiae</i> Basal Media
SRB	Sulphur Reducing Bacteria
SWA	Sea Water Agar

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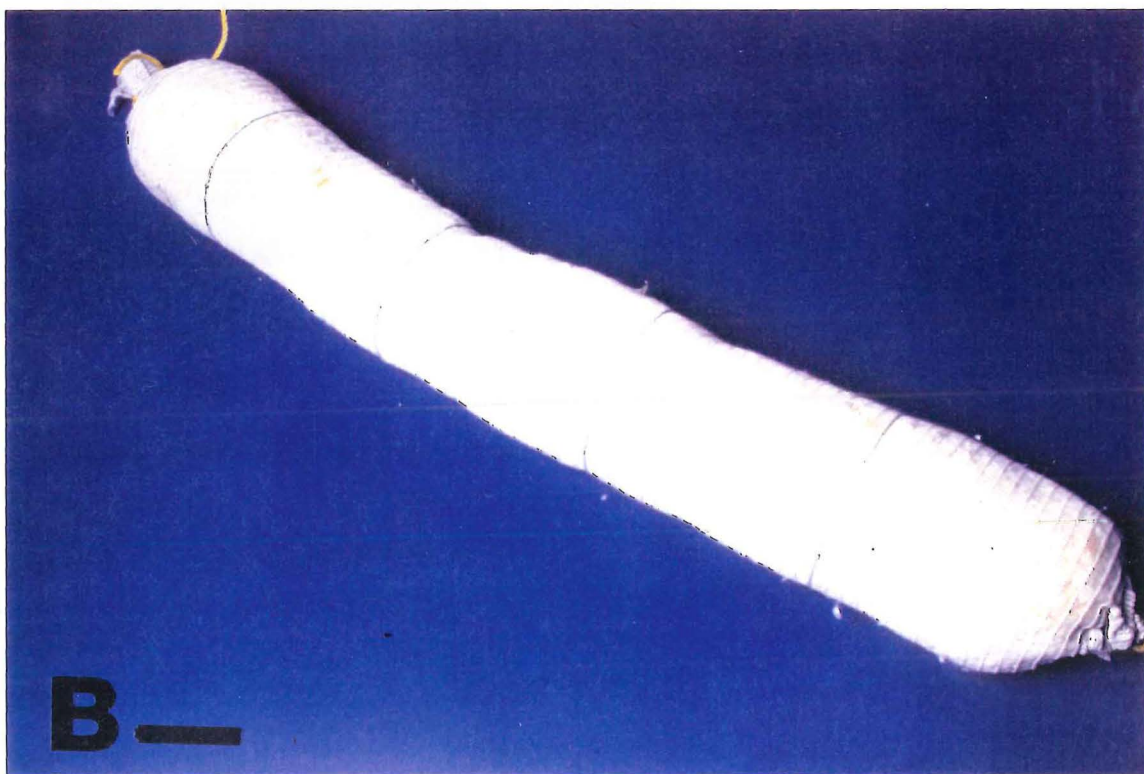
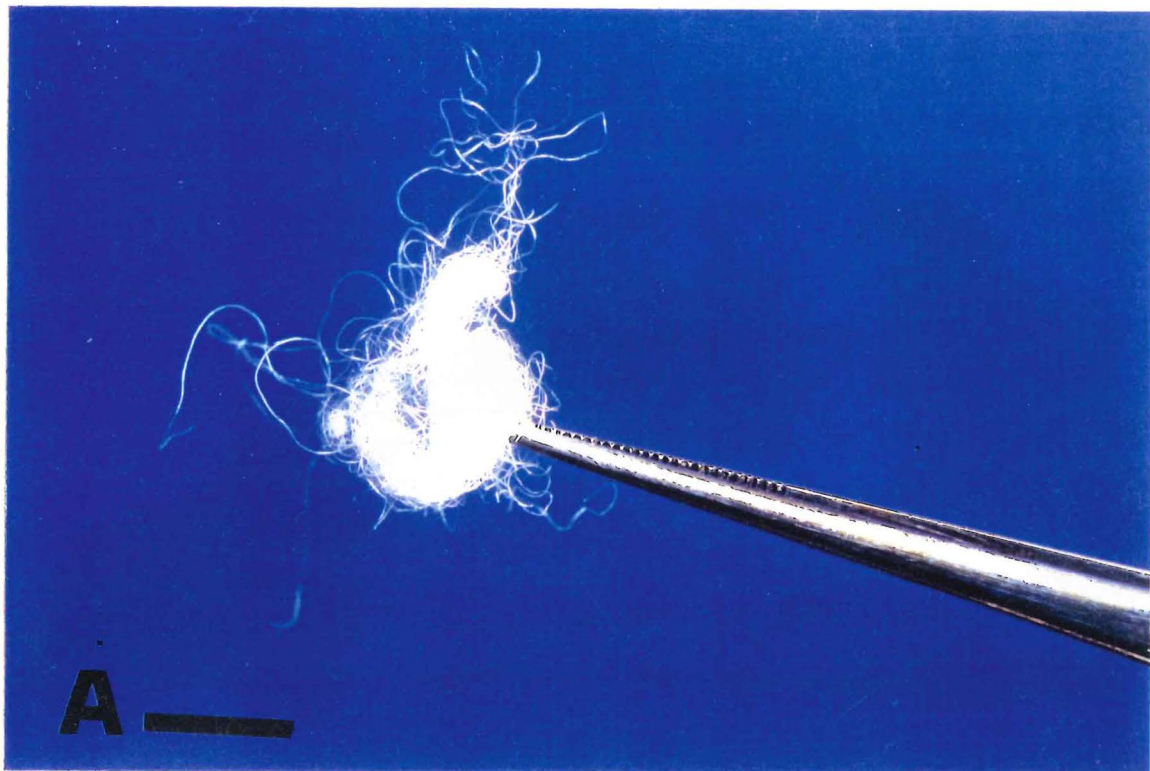


## 1. INTRODUCTION.

### 1.1 OIL SPILL SORBENTS.

About 3.5 million tonnes of oil are deposited into the world's oceans yearly. This represents approximately one tonne lost for every 1000 tonnes removed from the earth (Middleton, 1990). As most catastrophic or chronic pollution of oil in the marine environment occurs close to the coast during transport and refining, any large spillage of oil can have visible and ecological consequences. As a result of this one of the first concerns of any clean up operation is to contain the spill and remove the surface oil. A number of products known as sorbents have been developed to achieve this (SL Ross Environmental Research Ltd, 1991). Until recently the most efficient sorbents have contained petroleum derived compounds, which if not recovered or allowed to float free of their containing structures, may themselves become a marine pollutant. Recently the Wool Research Organisation of New Zealand (W.R.O.N.Z.) have developed Woolspill<sup>TM</sup> (Wool Research Organisation of New Zealand, 1992). This product involves the use of coarse short wool fibres 33-37  $\mu\text{m}$  in diameter, which are rolled into small balls, known as knops (Wool Research Organisation of New Zealand, 1992) (Figure 1). These knops can be easily manipulated into a number of products, such as booms, mattresses, and cushions which can be used to clean up oil spills (Donaghys, 1992; Wool Research Organisation of New Zealand, 1992). As wool is a natural product it is able to be broken down by microorganisms to yield amino acids, which in turn would be broken down to yield carbon dioxide and water. Biodegradability is desirable as wool knops can therefore be used separately from the containment fabric, by placing loose knops on to the surface of an oil slick, to adsorb any surface oil. This is an advantage, as it has been shown that uncontained wool knops are capable of absorbing more oil than knops contained within booms (Donaghys, 1992). After adsorption of any oil knops can then be removed from the seawater surface by vacuum or raking (Donaghys, 1992). Being biodegradable also means that Woolspill<sup>TM</sup> is more acceptable for dumping in landfills, than synthetic sorbents (Wool Research Organisation of New Zealand, 1992), although if coated in oil this is debatable.

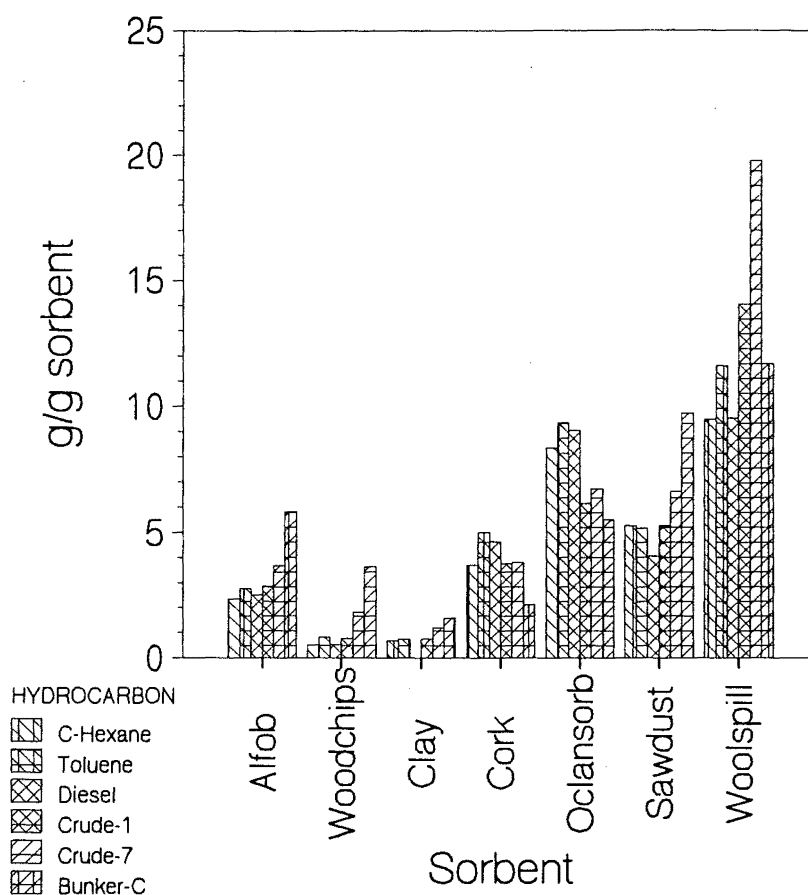
It has been shown in a number of studies that wool has a great affinity for viscous oils, such as crude and engine oils. Wool knops compare favourably with other organic sorbents, such as clay, cork, and sawdust, and with synthetic sorbents, such as polyurethane foams and powders (Figure 2) (SL Ross Environmental Research Ltd, 1991). Raw wool will adsorb up to eight times its own weight in oil, due to its natural oleophilic nature (Wool Research Organisation of New Zealand, 1992). That is its ability to trap oil with its scales, which in nature act as a trap for lanolin oil excreted by





**Figure 1. Woolspill™.**

Woolspill™ is composed of small balls of wool known as knops (A) (bar = 1 cm). Knops can be manipulated into a number of products such as booms, for the adsorption of oil (B) (bar = 10 cm). Due to the buoyant nature of wool oil booms, they are able to be used to adsorb marine oil spills (C).



**Figure 2. Average Initial Capacities of Organic Sorbents for Various Oils and Oil Derived Products.**

Woolspill<sup>TM</sup> was found to be one the most efficient organic sorbents, in adsorbing various oils, during evaluation trials in Canada (from SL Ross Environmental Research Ltd., 1991).

sheep. This gives a fleece its water repellency, and insulation (Wool Research Organisation of New Zealand, 1992). When wool is rolled into knops it has the ability to adsorb forty times its own weight in oil, depending on the oil's viscosity, this is because rolling the wool forms the fibres into a greater surface area and hence gives the oil increased access to fibres, and enhances the potential to adsorb (Donaghys, 1992). Oil is trapped by the scales of a wool fibre, rather than absorbed into the fibre in a blotting paper type action. This adsorbing action allows the wool knops to be recycled by wringing or hydroextraction of the oil from the wool knops.

## 1.2. THE STRUCTURE OF WOOL.

Most animals are more or less covered in fibres which are formed within follicles in the skin, and which grow protruding the skin. In sheep these fibres are known as wool. Wool fibres are a complex mixture of proteins known as  $\alpha$ -keratins. These proteins are also found in other anatomical parts of animals such as finger nails, horn, hooves, and quills. Hence forth  $\alpha$ -keratin will be referred to as keratin, unless stated otherwise (Postie *et al.*, 1988).

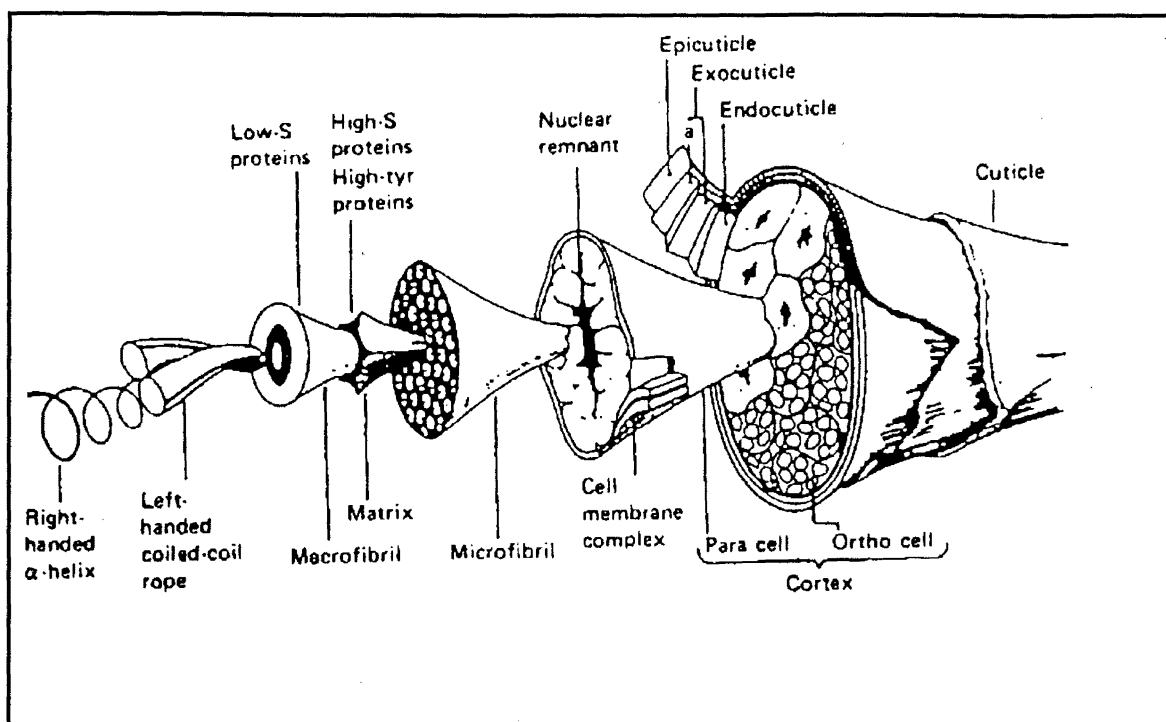
### 1.2.1. The Physical Structure of Wool.

The mean diameter of wool fibres varies between sheep breeds from 15  $\mu\text{m}$  to 50  $\mu\text{m}$ . In transverse section these fibres appear elliptical. The physical structure of a wool fibre can be divided into a number of different parts (Figure 3) (Hearle and Peters, 1963; Postie *et al.*, 1988).

The outer layer of the wool fibre is known as the scales or cuticle. These are flattened overlapping cells cemented to one another. The scales cover the main body of the fibre known as the cortex (Postie *et al.*, 1988).

The cortex is comprised of spindle shaped cells 500  $\mu\text{m}$  long and 50  $\mu\text{m}$  thick, known as cortical cells. These cells are cemented together by an intracellular membrane complex. This cement is different from the cortical cells in that it easily swells and is believed to be the site of some forms of wool breakdown caused by fatigue and abrasion. The cortex comprises approximately 90% of the wool fibre with the cement and scales comprising the additional 10% (Postie *et al.*, 1988).

The cortical cells are comprised of filamentous material called macrofibrils, which are 0.3  $\mu\text{m}$  thick and span the length of the cells. Within these macrofibrils are smaller structural units known as microfibrils. These microfibrils are the basic mechanical unit of the wool fibre. They have variable length and are approximately 7.5 nm thick, and occupy 50% of the fibre volume. Microfibrils consist of staggered rigid rods which comprise pairs of  $\alpha$ -



**Figure 3. The Structure of a Wool Fibre.**

The wool fibre can be divided into a number of structures. The two major components are the non-keratinous cuticle (or scales), and the keratinous cortex (from Postie *et al.*, 1988).



helical ropes interlocked along their lengths. These  $\alpha$ -helical ropes represent the molecular structure of keratin, and have a diameter of 1 nm (Postie *et al.*, 1988).

The chemical analysis of the fibres show two types of protein which differ in the amounts of sulphur they contain. The low sulphur containing protein occurs in the microfibrils, while the high sulphur containing protein occurs between the microfibrils. These high sulphur containing proteins are unable to form  $\alpha$ -helical material and therefore have no physical structure. The scales contain high sulphur proteins and are therefore devoid of microfibrils (Postie *et al.*, 1988).

In coarse wool fibres there is another part to the fibre. This is the medulla. This area is an axial stream of cells in the fibre's centre. This may range from a small amount of material which is not continuous, to a large core. The medulla unlike the cortex and scales does not contain a compact, dense structure, but contains a large number of vacuoles. This structure appears to stiffen the wool fibre (Bradbury, 1973).

Overall the physical structure of a wool fibre can be considered as a composite of filamentous material aligned parallel to the fibre axis and embedded in a matrix (Postie *et al.*, 1988).

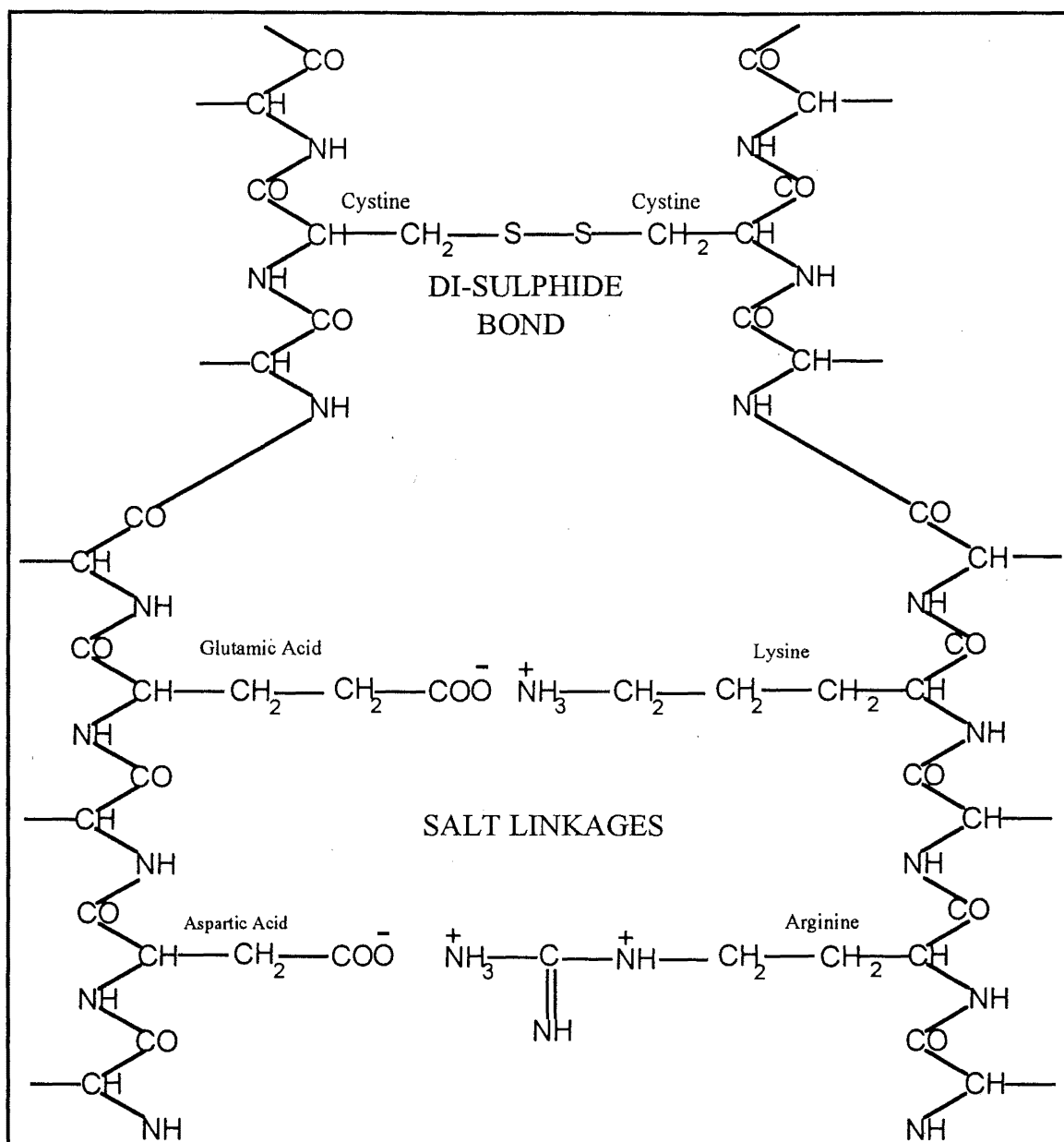
### **1.2.2. The Structure of Keratin.**

Keratin is a tough fibrous protein. Many of its mechanical, chemical, and biological properties can be attributed to its structure. The molecular structure of keratin is similar to that of other fibrous proteins in that it is made up of folded polypeptide chains which lie parallel to one another along the axis of the fibre. These fibres are held together via three important cross links, those of hydrogen bonding, salt linkages, and most importantly disulphide bonds (Mathison, 1964).

Keratin protein has been shown to contain a large number of highly polar peptide linkages which give rise to a number of inter and intra-molecular hydrogen bonds. These bonds cause the keratin fibres to be supercontracted into a configuration known as  $\alpha$ -keratin. When these hydrogen bonds are broken the configuration is known as  $\beta$ -keratin (Matthews, 1967).

Salt bridges link the free basic amino and acidic carboxyl groups when a dicarboxylic or diamino acid is built into the polypeptide chain, such as between glutamic acid and lysine residues. Salt linkages are thought to play a role in impeding the digestion of keratin by enzymes (Mathison, 1964).

Probably the most important cross link in keratin that prevents enzymatic digestion, is that formed by the presence of disulphide bonds (Figure 4). This is caused



**Figure 4. Important Cross Links in Keratin.**

Disulphide bonds, and salt linkages are thought to be important in preventing keratin degradation by microorganisms. Disulphide bonds are formed due to the oxidation of sulphide groups in cysteine residues upon neighbouring polypeptide chains. Salt linkages form to link the free amino and acidic carboxyl groups, when a dicarboxylic or diamino acid is built into neighbouring polypeptide chains (adapted from Matthews, 1967).



by the presence, in high quantities (12-16%), of the amino acid cysteine within the protein. Cysteine contains sulphur in the form of a sulphide group which can form disulphide bridges between each other, thereby linking two cysteine amino acids in the keratin fibre (Hearle and Peters, 1963), to form a cystine molecule (resulting from the oxidation reaction between the sulphydryl groups) (Martin, 1990). Therefore a keratin fibre can be considered as a network of polypeptide chains linked together by disulphide bridges formed between cystine amino acids (Matthews, 1967).

### 1.3. THE MICROBIAL DEGRADATION OF KERATIN.

As keratin is a physically robust protein, there are relatively few keratinolytic microorganisms described, even though many keratinophilic microorganisms have been isolated (Mathison, 1964). The difference between keratinophilic and keratinolytic microorganisms are the action each type of microorganism has on the keratin fibre. For example a number of microorganisms are capable of growing on keratin, however many do not possess the ability to breakdown and degrade the protein. These types of microbes are referred to as keratinophilic. Microorganisms which are capable of degrading keratin fibres are referred to as keratinolytic (Mathison, 1964).

Keratin degradation by microorganisms has been extensively studied with a view to elucidating the ecology of dermatophytes in respect to human and animal health. Dermatophytes are keratinolytic microorganisms which cause infections in keratin containing appendages of animals, such as skin, hair, and nail (Howard, 1983). Most of the keratinolytic microorganisms recorded are fungi, although a number of bacterial species have been identified.

#### 1.3.1 Fungal Species.

The ability of fungi to degrade keratin is quite common, which explains the significant role played by these organisms in the natural degradation of keratin (Rajak *et al.*, 1991). The isolation of these organisms from soil can be done by the simple Toma-Karling-Vanbreusegham (To-Ka-Va) method of hair-baiting, where a keratin containing substance, such as hair or wool, is buried in soil either *in vivo* or *in vitro* (Benedek, 1962; Mathison, 1964). As a result a large number of fungi capable of colonising and attacking keratin have been isolated from a number of different parts of the world (Pugh and Mathison, 1962; Marples, 1965; Pugh and Hughes, 1975; Ulfig, 1983; Al-Musaliyam, 1990; Ulfig and Ulfig, 1990; Rajak *et al.*, 1991; Garg, 1992; Finotti *et al.*, 1993).

Although soil dwelling keratinolytic fungi have been extensively studied, very little investigation into the keratinolytic flora of aquatic habitats has been undertaken (Kohlmeyer and Kohlmeyer, 1979). Some freshwater fungi such as species from the

genera *Chrysosporium* and *Trichophyton* have been isolated from polluted freshwater sediments in Poland (Ulfig, 1983), the numbers of keratinolytic fungi isolated were found to correspond to the degree of pollution in the river (Ulfig and Ulfig, 1990). Although no investigation into the occurrence of keratinolytic fungi in marine sediments has been undertaken, a number of keratinolytic fungi have been isolated from coastal soils (Pugh and Mathison, 1962; Pugh and Hughes, 1975). In studies in British Columbia, Canada, no keratinolytic fungi were isolated from below the high water mark, and the isolation of keratinolytic fungi above this mark such as *Ctenomyces serratus* was suggested to occur due to the preening of sea birds, as *C. serratus* has been isolated from the preening glands and feathers of living birds (Pugh and Mathison, 1962). The absence of keratinolytic fungi from below the high water mark was thought to be attributed to the adverse effect sea water has on these organisms (Pugh and Hughes, 1975). Although no obligative or facultative marine fungi have been found that are capable of degrading keratin, a marine fungus *Abyssomyces hydrozicus* has been isolated which is capable of degrading keratin-like annelid tubes of sand inhabiting worms (Kohlmeyer and Kohlmeyer, 1979).

### 1.3.2. Bacterial Species.

Very few keratinolytic bacteria have been isolated, compared to fungi. The majority of keratinolytic bacterial species studied have been the actinomycetes. One of the first known keratinolytic bacteria isolated and studied was the soil bacterium *Streptomyces fradiae*. This bacterium was shown to degrade a number of keratin containing substances, including wool (Noval and Nickerson, 1959), subsequent studies have focused on enzymes liberated by *S. fradiae* to degrade keratin, and their mechanisms (Nickerson and Noval, 1961; Dobson and Bosely, 1963; Nickerson *et al.*, 1963; Young and Smith, 1975; Brady *et al.*, 1987; Carter *et al.*, 1987; Kunert and Stransky, 1988; Katuzweska, 1991; Shina *et al.*, 1991). Much of today's knowledge about bacterial keratinolytic activity can be attributed to these studies. Other *Streptomyces* species are known to be keratinolytic, however *S. fradiae* is considered the most keratinolytic of this genus, as it is the only species which completely solubilises keratin (Noval and Nickerson, 1959; Nickerson *et al.*, 1963; Brady *et al.*, 1987; Katuzweska, 1991).

Another soil actinomycete known to be keratinolytic is the actinoplanete *Pilimelia* (Karling, 1954; Gaertner, 1955; Kane, 1966; Tribe and Abu El-Soud, 1979; Vobis, 1989, 1992). However, compared to *Streptomyces* this genera has been relatively unstudied, this is mainly due to their slow growth (Walker and Colwell, 1975; Vobis, 1989), and in the past isolates have been confused with fungi, especially chytrids (Tribe and Abu El-Soud, 1979). These bacteria are so keratinolytic that they are only able to be isolated through a hair baiting technique (Couch, 1949), similar to the To-Ka-Va method for the isolation of keratinolytic fungi. Other soil bacteria isolated which are capable of

degrading keratin include a species tentatively identified as a *Cytophaga* (Napier, 1966), but later identified as a *Lysobacter* species (Reichenbach, 1992).

Bacterial dermatophytes, like fungal ones, have also come in for particular investigation. For example *Dermatophilus congolensis*, an unusual gram positive bacterium (Stackenbrandt *et al.*, 1983), has had its keratinolytic ability studied due to its production of exoenzymes capable of degrading keratinised tissues, causing pitted keratolysis, a disease of the palms and feet in humans, and an economically important disease in sheep and cattle (Hanel *et al.*, 1991).

Today keratinolytic bacteria are being studied as sources of keratinolytic enzymes capable of being used in biotechnological processes, such as for the removal of hair from skins of slaughtered animals in the tanning industry (Lewis, 1981; Ward, 1983; Brady *et al.*, 1987; Outtrup, 1990), or turning keratinous waste material into dietary supplements for animal feed (Katuzweska, 1991). For example a keratinolytic strain of *Bacillus lichenformis* was isolated from poultry feathers (Williams and Shih, 1989), with a view to producing a thermophilic poultry waste digester capable of utilising feathers from poultry farms, and turning them into a protein supplement for animals (Williams *et al.*, 1990; Xiang *et al.*, 1992).

### 1.3.3. Wool Degradation.

Wool is a very important fibre to humans. Therefore the effect of microbial damage on wool has been extensively studied in an effort to understand the mechanisms, and potential for control of fleece damage in sheep, and in processed wool.

The presence of large numbers of microorganisms in fleece wool has been reported (Dye, 1964; Dye and Rothbaum, 1964; Rothbaum and Dye, 1964; Lewis, 1981). It has often appeared that these bacteria were growing not on the wool keratin, but rather on grease, such as lanolin, and other contaminants of the wool. Most economically important damage done to fleece wool is through staining with the accumulation of particular pigments by the microorganism, rather than through fibre degradation (Lewis, 1981). However some microorganisms are known to both degrade and stain the wool fibre. For example, Pink Rot of wool occurs due to *Bacillus vulgatus* or *Bacillus subtilis* which stains the wool pink (Henderson, 1968; Lewis, 1981), and also degrades the material between the cortical cells of the wool fibre, causing fibrillation and release of cortical cells (Molyneux, 1959). A number of pseudomonads are also known to cause staining and associated rotting. One of the most serious of these diseases is green banding caused by *Pseudomonas aeruginosa*. This disease is known as green banding due to the characteristic green pigment which develops on the fleece wool (Henderson, 1968). These bacteria also cause rotting of the wool fibre, as well as myiasis (dermatophytic disease) of the sheep skin, at the base of the fibre (Burrell *et al.*, 1982;

Burrell, 1990). The weakening of the wool fibre has been shown in tensile strength tests performed on wool exposed to this bacterium (MacDiarmid and Burrell, 1993).

Bacteria are not the only contributing microorganisms in fleece disease. Fungi are also known to cause fibre degradation (Lewis, 1981). For example *Peronellaea glomerata* has been isolated and shown to be the causative agent in the phenomenon known as Black Fungus Tip, where the staple of the wool is coloured tarry black by the black pigmented fungal hyphae (Mulcock, 1959).

Processed wool is also susceptible to microbial degradation, when used in textiles. Damage caused by microorganisms in textiles is commonly referred to as mildew. This terminology is fundamentally incorrect however, as most damage is not caused by fungi, as this term infers, but rather through bacterial attack (Lewis, 1975). Microorganisms generally known to attack processed wool are generally those already mentioned, such as bacilli, pseudomonads, and actinomycetes, as well as fungi, such as *Penicillium* and *Aspergillus* (Lewis, 1981).

#### **1.3.4. Keratinases and the Study of Keratinolytic Activity.**

The proteolytic ability of microorganisms is dependant upon the ability of the microorganism to produce a proteolytic enzyme capable of attacking the protein fibre and cleaving it into peptides, which can then be assimilated into living cells (Lewis, 1981). When the protein is keratin, the proteolytic enzyme is known as a keratinase.

##### **1.3.4.1. Bacterial Keratinases.**

The ability of bacteria to produce keratinases is disputed. The ability of *S. fradiae* to degrade wool in basal salt culture, containing wool as sole carbon and nitrogen source was attributed to the bacterium producing an enzyme capable of degrading keratin, and hence the enzyme was named keratinase (Noval and Nickerson, 1959).

Other studies however, showed that cell free filtrates of the bacterium were able to degrade wool, but were unable to produce soluble sulfhydryl compounds. Soluble sulfhydryl compounds are generally considered to be present during keratin degradation as a result of the disruption of disulphide bonds (Mathison, 1964).

Additional studies on the enzymes produced by *S. fradiae* have shown that they are proteases, and are also induced by other protein substrates such as elastin, and casein (Moriyama *et al.*, 1967). Scanning electron microscopy has shown that when wool is degraded by *S. fradiae*, the non-keratinous proteins of the wool fibre are degraded, releasing keratin containing cortical cells, and therefore degrading the wool fibre (Brady *et al.*, 1990). This indicates that the term keratinase may be inappropriate to describe the enzymes of *S. fradiae*.

A feather degrading *B. lichenformis* strain has also been studied for keratinase activity. The degradation of steam treated feathers was studied by monitoring the increase in concentration of free amino acids, and soluble sulfhydryls (Williams *et al.*, 1990). However, like *S. fradiae*, the enzymes from *B. lichenformis* were found to be unable to produce soluble sulfhydryl compounds. The enzymes were characterised and also found to be strong proteases capable of degrading the proteins elastin and collagen (Xiang *et al.*, 1992). No electron microscopy studies have been performed upon this bacterium.

#### **1.3.4.2. Fungal Keratinases.**

In fungi the production of "true" keratinases appears to be more certain. It has been observed that dermatophytes are able to degrade keratin by first breaking the disulphide bridges. This is achieved by the fungus oxidising the cysteine in the keratin fibre with the assistance of excreted sulphite, leaving behind inorganic sulphate as the main excretory product. This process has also been observed in non-dermatophytic fungi (Malviya *et al.*, 1993a, b). Sulfitolysis allows the fungus to denature the protein, and allows access to fungal proteases to cleave the peptide for assimilation by the fungus.

The importance of the breakage of disulphide bonds in degrading keratin has been seen in studies where the keratin substrate is treated with reducing agents. This increases keratin degradability.

### **1.4. MARINE MICROBIOLOGY.**

The world's oceans, which cover 70% of the earth, can be considered a milieu of microbial growth. The biology of this milieu can be considered to be dominated by the prokaryotes and small eukaryotic algae, fungi, and protozoa (Floodgate, 1984).

Although the numbers of phytoplankton and zooplankton are well recorded, it was not always realised how large the bacterial, and fungal contribution to the total marine biomass is. Although bacterial numbers vary from place to place, and time to time, coastal waters may typically be said to contain around  $10^{11}$ - $10^{12}$  organisms per  $m^3$ . It is therefore of no surprise that in any sample of sea water there are microorganisms capable of degrading a number of different biotic and abiotic compounds, some of which are quite complex (Austin, 1988)

This ability to degrade complex molecules can be attributed to the fact that most organic compounds produced have a structure which is too large to be readily assimilated into a cell. As a result most eukaryotes and prokaryotes have developed an alternative strategy for the assimilation of complex compounds. This strategy involves the use of hydrolytic enzyme which are secreted outside the cytoplasmic membrane, and

which hydrolyses the large macromolecules in close proximity to the cell. The resulting low molecular products are then able to be actively transported across the cell membrane for use by the cell, within the cytoplasm. These enzymes are known as extracellular enzymes, exoenzymes, or ectoenzymes, and may be associated with the cell surface, or be free floating so that the enzyme is actively liberated and is dissolved into the surrounding water, or adsorbed to surfaces other than that of the producer (Chrost, 1987).

#### 1.4.1 Marine Bacteria.

Marine bacteria possess a number of different types of enzymes which have been well studied. The distribution of amylase, lipase, and protease producing bacteria in surface and sub-surface water off the coast of Sweden has been studied (Kjelleberg and Hakansson, 1977). Between 43-100% of aerobic heterotrophs produced lipases, 47-100% produced proteases, and 17-88% produced amylases. Similar studies on the enzymatic capabilities of sediment dwelling bacteria off the coast of the USA have also been studied (Nitkowiski *et al.*, 1977). It was found that a similarly high proportion of bacteria were lipolytic and proteolytic. Other complex molecules known to be broken down are chitin, mannan, and glucan (Austin, 1988). Knowledge of keratin degradation by marine bacteria is negligible.

#### 1.4.2. Marine Fungi.

The other component of marine microflora are the marine fungi. Compared to the marine bacteria, the marine fungi have been little studied, and hence their role in the marine ecosystem has is only partially understood (Kohlmeyer and Kohlmeyer, 1979).

There are basically two types of marine fungi. There are the obligate marine fungi which are only able to grow and sporulate in marine or estuarine environments. The other type of marine fungi are the facultative marine fungi which occur not only in marine habitats, but also in terrestrial habitats. The majority are obligately marine. Marine fungi are represented by most divisions of fungi with most being *Ascomycetes*, *Basidiomycetes*, or *Deuteromycetes* (Kohlmeyer and Kohlmeyer, 1979).

There is little knowledge about marine fungi enzymology, and physiology (Moss, 1986). However, it is known that like their terrestrial counterparts, marine fungi are capable of producing a number of enzymes including amylases, proteases, chitinases, and cellulases (Moss, 1986). The ability of marine fungi to produce enzymes capable of attacking proteins (proteases) have been demonstrated in a number of studies. This ability seems to be relatively common within this group of fungi. One study showed that 13 out of 14 species of marine fungi had the proteolytic enzyme gelatinase (Pisano *et al.*, 1964). Little is known about the degradation of keratin by marine fungi.

### 1.4.3. Biofilms.

Biofilms are composed of microorganisms immobilised within an organic polymer matrix, upon a surface. Biofilms develop upon virtually all surfaces which are immersed in the marine environment, and are particularly prevalent in areas of flowing water, where a continuous supply of nutrients is available for attached microbes. The subject of biofilms has been reviewed by Marshall (1992).

For microbes, biofilms are advantageous as the bacteria can form integrated communities, where the resident bacteria have access to nutrients from the surrounding water, and organic compounds excreted by other bacteria, as well as protection from antibiotics, chlorination, and other antimicrobial products.

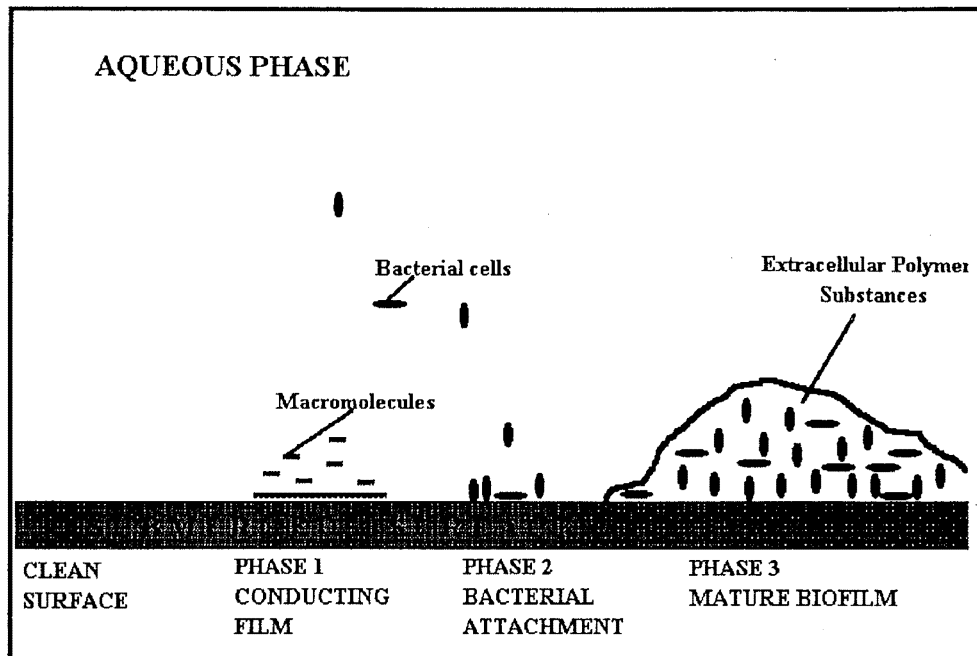
Biofilm development occurs in three phases (Figure 5). Phase 1 involves the adsorption of macromolecules, and hydrophobic molecules onto the submerged surface, to form a conditioning film. These conditioning films alter the charge and free energy of the surface allowing bacterial adhesion. Phase 2 occurs as bacteria attach to the surface. This occurs via the activation of a number of genes in bacteria which allow the bacteria to act differently upon surfaces than in aqueous phases. For example bacteria may produce surfactants, organic molecules, or change their flagellation to allow their cells to attach. Phase 3 occurs in a mature biofilm where the bacteria metabolise macromolecules bound to the surface, and reproduce. The progeny of these bacteria are either released into the aqueous phase to form microcolonies or slowly migrate away from each other, along the surface, to divide. As a biofilm matures, extracellular polymeric substances are produced, embedding the bacteria. Biofilms are not a static environment, and are turned over by the constant sloughing off, and building up of the biofilm.

## 1.5. AIMS AND EXPERIMENTAL STRATEGY.

The aim of this study was to investigate the microbial degradation of wool in the marine environment, so that an understanding of the processes that occur in the degradation of wool oil booms may be found.

Wool degradation in the marine environment was analysed by:-

1. The establishment of cultures of microbes from sea water enriched on woollen substrates *in vitro*, so that biochemical indicators of wool degradation could be monitored, and wool degradation could be observed microscopically.
2. The development of a plate assay for rapidly screening possible keratinolytic bacteria from the marine environment.



**Figure 5. Biofilm Establishment.**

The three phases of biofilm establishment on submerged surfaces (adapted from Marshall, 1992).



3. The placement of a wool oil boom in Lyttleton Harbour to enrich the microflora responsible for the degradation of wool in the marine environment, so that they could be isolated, and identified, and their action observed using microscopy.

## 2. MATERIALS AND METHODS.

### 2.1. STERILISATION TECHNIQUES.

#### 2.1.1. Keratin.

Keratin containing substances such as wool knops, woollen fabric, and Keratin Azure (Sigma), were sterilised using ethylene oxide, as more traditional sterilisation techniques, such as autoclaving, has been shown to partially degrade keratin (Noval and Nickerson, 1959). Sterilisation was performed by the Central Sterile Supplies Depot of the Canterbury Area Health Board. Samples were exposed to ethylene oxide for 8 hours, in a pressurised vessel. After exposure, ethylene oxide was evacuated from the vessel, and the sample aired in sterile conditions for 13 hours.

Ethylene oxide sterilised materials were tested for sterility by placing upon Nutrient Agar (Difco) and incubating at 25°C for 7 days.

#### 2.1.2. Sea Water.

Sea water was sterilised using filter sterilisation. For large quantities (greater than 10 ml) a sterile 47 mm millipore filter holder containing a 0.8 µm prefilter (Gelman), a 10 µm polypropylene filter (Gelman), and a 0.22 µm filter (Gelman) was used. For smaller samples (less than 10 ml) a sterile 0.22 µm Acrodisc (Gelman) unit was used.

### 2.2. ASSAYS FOR WOOL DEGRADATION.

#### 2.2.1. Keratinolytic Bacteria.

Two bacterial species of known keratinolytic activity were purchased, so that they may be used as positive controls. *Streptomyces fradiae* (ATCC 14544) was obtained from the American Type Culture Collection, and revived according to supplied methodology. This culture was maintained on Potato Dextrose Agar (PDA) (Difco).

A *Lysobacter* species (NCIMB 9497) was obtained from the National Collection of Industrial and Marine Bacteria. This culture was revived according to the methodology supplied. This culture was maintained on LPC agar (Reichenbach, 1992). This media consisted of 0.25% (w/v) Yeast Extract (Difco), 0.5% (w/v) Tryptone (Difco), 0.1% (w/v) glucose, 1.5% (w/v) bacteriological agar, in distilled water.

Cultures of both bacteria were grown at 25°C.

#### 2.2.2. Biodegradation of Wool *In vitro*.

Cultures containing Keratin Azure, and wool knops, were established for microbes in sea water, and keratinolytic bacteria, so that the degradation of woollen substrates could be monitored in the laboratory.

### 2.2.2.1. Use of Keratin Azure as a Model for Wool Degradation.

Keratin Azure comprises lambs wool which has been dyed with Azure, a blue biological stain. As the wool fibre is degraded the stain is released into the surrounding solution (Sigma, personal communication). This allows the rate of wool degradation to be monitored qualitatively, and quantitatively. Keratin Azure has been used previously to examine the keratinolytic activity of a bacterium, when used with *Dermatophilus congolensis* (Hanel *et al.*, 1991).

#### 2.2.2.1.1. Enrichment Cultures for Marine Microbes able to Degrade Keratin Azure.

Mixed cultures of marine microbes growing on Keratin azure were established. This was achieved by adding 0.3 g of Keratin Azure and 200 ml of sea water, freshly collected from Lyttleton harbour, to a 250 ml conical flask.

A control flask was prepared by adding 0.3 g of Keratin Azure, to 200 ml of sterile sea water.

Cultures of *S. fradiae* and *Lysobacter* growing on Keratin Azure were established in 250 ml conical flasks. Each flask contained 0.3 g of sterile Keratin Azure, and 200 ml of carbon and nitrogen free basal salts media. The *S. fradiae* basal salts media (SFBM) was adapted from Noval and Nickerson (1959), and consisted of 0.15% (w/v) dibasic potassium phosphate ( $K_2HPO_4$ ) (Sigma), 0.0025% (w/v) magnesium heptahydrate ( $MgSO_4 \cdot 7H_2O$ ) (BDH), 0.0015% (w/v) ferric sulphate heptahydrate ( $FeSO_4 \cdot 7H_2O$ ) (BDH), 0.0005% (w/v) zinc sulphate heptahydrate ( $ZnSO_4 \cdot 7H_2O$ ) (BDH), and 0.0025% (w/v) anhydrous calcium chloride ( $CaCl_2$ ) (BDH), in distilled water. *Lysobacter* basal salts media (LBM) (Martin and So, 1969) consisted of 0.1% (w/v)  $K_2HPO_4$ , 0.05% (w/v)  $MgSO_4 \cdot 7H_2O$ , 0.02% (w/v) ferric chloride ( $FeCl_2$ ) (BDH), 0.01% (w/v) sodium chloride (NaCl) (BDH), in distilled water (pH 8.2).

*S. fradiae* or *Lysobacter* were inoculated into the conical flasks by preparing an inoculating solution. This inoculum was created by inoculating 20 ml centrifuge tubes containing 15 ml of Potato Dextrose Broth (PDB) (Difco) or *Lysobacter* culturing broth (PEP) (Reichenbach, 1992), with a loopful of bacteria taken from a plate culture. PEP broth consisted of 1% (w/v) Casitone (Difco), and 0.1% (w/v)  $MgSO_4 \cdot 7H_2O$ , in distilled water (pH 7.2). The tubes were incubated at 25°C for 7 days. After incubation the tubes were centrifuged at 8600 g for 15 mins in a Sigma 2K15 Centrifuge. After centrifuging the supernatant was discarded and the pellet was washed by resuspending in either SFBM or LBM depending on the culture. This solution was again spun down in a Sigma 2K15 Centrifuge and the pellet washed twice more. The pellet was resuspended in 5 ml of LBM or SFBM. These solutions were used to inoculate the conical flasks.

Control flasks were established using 200 ml of either SFBM or LBM, and 0.3 g of Keratin Azure in 250 ml conical flasks. No bacterial inoculum was added to these flasks.

All flasks were incubated at 18°C in a Gallencamp Orbital Incubator at 150 rev/min.

#### **2.2.2.1.2. Monitoring of Keratin Azure Biodegradation.**

Flasks containing Keratin Azure were sampled at 4 weekly intervals for the mixed cultures of marine microbes, and at weekly intervals for the cultures of the keratinolytic bacteria, *S. fradiae*, and *Lysobacter*. Three ml of liquid were removed from each flask. This was placed into a 10 ml centrifuge tube, which was centrifuged at 8600g for 30 mins in a Sigma 2K15 Centrifuge to remove any residual Keratin Azure and bacterial cells which may have interfered with spectrophotometry.

The sample was then placed within a quartz cuvette, and the absorbance read at 595 nm on a Hitachi U-2000 Spectrophotometer (Hanel *et al.*, 1991), so that the level of stain liberation could be monitored. The sample was compared to a blank of sterile sea water.

Fibres of Keratin Azure were removed from the cultures at sampling times and examined. Single fibres were removed only as not to disturb the rate of degradation, and stain release. The fibres were then observed and photographed under an Olympus BH-2 Light Microscope, by wet mounting in distilled water on a glass microscope slide.

#### **2.2.2.2. Biodegradation of Wool.**

Cultures containing wool knops, were established for microbes in sea water, and keratinolytic bacteria, so that wool degradation could be monitored in the laboratory, by light microscopy, and the release of biochemical indicators.

##### **2.2.2.2.1. Enrichment Cultures for Marine Microbes able to Degrade Wool.**

Cultures of marine microbes in sea water, and keratinolytic controls, were established as described previously for the Keratin Azure cultures in section 2.2.2.1., except that 1g of sterile wool knops supplied by WRONZ, were used in place of Keratin Azure.

12 ml samples from each flask culture were removed at the same time intervals, as for Keratin Azure. These samples were then placed in 20 ml centrifuge tubes and centrifuged at 8600 g for 10 mins to remove any wool and bacterial cells from the solution. The supernatant was then filtered through a 0.22 µm Acrodisc to remove any turbidity. These samples were then tested for free amino groups, and soluble sulfhydryl concentrations, as these are considered as indicators of wool degradation.

#### 2.2.2.2.2. Free Amino Group Concentrations.

Samples from the cultures which had been centrifuged and filtered, were tested for their free amino group concentration. Two ml of each sample were placed into separate test tubes, with 3 replicates. These samples were tested with a ninhydrin reagent, which was adapted from that of Rosen (1957). This reagent was prepared fresh and stored in a brown bottle. It consisted of 2% (w/v) ninhydrin (BDH), 3% (w/v) hydrindantin (Sigma), 75% (v/v) ethylene glycol monoethyl ether (Sigma), and 25% (v/v) acetate buffer (pH 5.5). To each test tube 2 ml of the ninhydrin reagent was added. The test tubes were heated in a boiling water bath for 15 mins. After which they were removed, and cooled to room temperature before 3 ml of 50% ethanol was added to stabilise colour change.

The absorbance of each sample was measured using a Hitachi U-2000 Spectrophotometer at 570 nm, using a reference blank of sterile sea water for cultures containing sea water, and SFBM or LBM for the culture of the keratinolytic bacteria, and their equivalent controls. These blanks were treated in the same way as culture samples.

Various concentrations of leucine (Sigma) (Shih *et al.*, 1977), were dissolved in sterile sea water, SFBM, or LBM, and used to construct a calibration curve for the estimation of free amino group concentrations. These samples were tested using the same method as the samples removed from the flask cultures.

#### 2.2.2.2.3. Soluble Sulphydryl Concentrations.

Samples from the cultures which had been centrifuged and filtered, were tested for their soluble sulphydryl concentration (Ellman, 1959). Two ml of each sample was placed into a test tube, with 3 replicates. To each test tube 3 ml of 0.5 M  $K_2HPO_4$  was added, along with 0.4 ml of Ellman's reagent. Ellman's reagent comprised of 40 mg of 5,5'-Dithiobis-(2-nitrobenzoic Acid) (DTNB) (Sigma), and 1 mM of Ethylenediaminetetraacetic Acid (EDTA) (Sigma), in 10 ml of potassium phosphate buffer (pH 8.0).

After 10 mins a yellow solution formed which was filtered through Whatman #1 filter paper. The absorbance of the resulting filtrate was read at 412 nm on a spectrophotometer. The sample was referenced against a blank of sterile sea water for cultures containing sea water, and LBM or SFBM for *Lysobacter* and *S. fradiae* cultures, and controls.

A calibration curve was constructed using reduced glutathione (Sigma) (Shih *et al.*, 1977), dissolved in either sterile sea water, or LBM, or SFBM. The samples were tested using the procedure used for detecting soluble sulphydryls in the culture media.

#### 2.2.2.2.4. Microscopic Examination of Wool Fibre Damage.

At every sampling single wool fibres were removed from the flask cultures and examined under light microscopy for microbial growth and fibre damage.

### 2.2.3. Development of a Plate Assay for Keratinolytic Activity.

A keratinolytic plate assay was developed for the rapid screening of microbes with the ability to degrade keratin. This was achieved by using soluble keratin protein (KS) in a method adapted from that of Wawrzekiewicz *et al.* (1987).

#### 2.2.3.1. Preparation of Soluble Keratin Protein (KS).

KS was produced by dissolving 10 g of clean poultry feathers in 500 ml of dimethyl sulfoxide (DMSO) (BDH), in a 1 L round-flat bottomed flask. This was heated upon an element at 100°C in a reflux condenser, for 2 hours with occasional stirring. After this time the solution was filtered through 2 layers of muslin to separate undissolved feathers and DMSO containing keratin.

The keratin containing DMSO was divided in half and placed into 2 1 L Schott bottles. Keratin protein was precipitated out of the DMSO with 2 volumes of supercooled acetone (BDH) (-80°C) per volume of DMSO and keratin solution. A thick caseous (cheese-like) precipitate formed, and was enhanced by leaving the solution in a refrigerator for 15 hours.

The keratin precipitate was separated from the remaining solution by centrifuging at 6000 g for 30 minutes in a Hereaus Varifuge 20R3 centrifuge. The supernatant was decanted and the sediment washed in 4 volumes of distilled water per volume of sediment, and centrifuged at 6000g for 30 minutes. After washing the supernatant was discarded and the sediment transferred into a beaker and stored in a refrigerator until required.

#### 2.2.3.2. Degradation of KS by keratinolytic Microorganisms.

The ability of KS to be degraded by keratinolytic bacteria was tested by plating *S. fradiae* onto agar containing KS as sole carbon and nitrogen source.

A carbon and nitrogen free agar SFBA was developed for *S. fradiae* which contained 0.15% (w/v)  $K_2HPO_4$ , 0.0025% (w/v)  $MgSO_4 \cdot 7H_2O$ , 0.0015% (w/v)  $FeSO_4 \cdot 7H_2O$ , 0.0005% (w/v)  $ZnSO_4 \cdot 7H_2O$ , and 0.0025% (w/v)  $CaCl_2$ , and 2% (w/v) Bacteriological Agar (Difco), in distilled water. This was used as a base, and a KS containing overlay was poured above. The overlay comprised 5% (w/v) KS mixed into distilled water. This solution was mixed using an Ultra-Turrax Homogeniser for 1 min. The solution was then ultrasonificated using a Megason Sonic Disintegrator for 5 mins, to further homogenise, and also sterilise the solution. Four ml of the sterile KS solution was added to 4 ml of molten SFBA in a sterile test tube. This solution was vortexed and

poured over the SFBA base and allowed to solidify. The KS overlay forms an opaque layer over a clear base. This media was stab inoculated with *S. fradiae*, and incubated at 25°C for 14 days, after which the plates were examined for zones of clearing in the opaque layer, indicating hydrolysis of KS.

#### **2.2.3.3. Degradation of KS by Marine Microorganisms.**

A similar carbon and nitrogen free agar, and KS overlay was developed for marine bacteria and fungi. This media was known as Marine Keratinolytic Agar (MKA), and consisted of a base of Sea Water Agar (SWA). SWA consisted of 2% (w/v) Bacteriological Agar (Gibco) in Artificial Sea Water (ASW) (Reichenbach, 1992). ASW comprised 2.47% (w/v) NaCl, 0.07% (w/v) potassium chloride (KCl) (Sigma), 0.63% (w/v) MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.46% (w/v) magnesium chloride hexahydrate (MgCl<sub>2</sub>·6H<sub>2</sub>O) (BDH), 0.01% (w/v) CaCl<sub>2</sub>, in distilled water. A KS overlay was produced as described previously except that for this media 5% (w/v) KS was mixed with ASW, and added to molten SWA. This media was tested with marine bacteria that had been isolated from wool oil booms placed in Lyttleton Harbour during preliminary studies. Marine bacteria were inoculated onto this media using stab inoculation. These plates were incubated at 18°C, for 14 days. Degradation of KS was indicated as previously for *S. fradiae*.

### **2.3. BIODEGRADATION OF WOOL *IN SITU*.**

#### **2.3.1. Wool Boom Placement and Sampling.**

A Woolspill<sup>TM</sup> wool oil boom (Donaghys), 1m in length was placed in Lyttleton Harbour, and secured to the Lyttleton Port Company Oil Wharf, so that wool degrading marine microbes may be enriched, and microscopic examination of wool damage could be performed. The boom consisted of wool knops encased in a polypropylene mesh. The boom was attached to the wharf so that the boom would remain in sea water at both high and low tides. The boom was sampled at monthly intervals, by removing wool knops and placing them in a sterile 1 L Schott bottle containing sterile sea water to prevent desiccation of the samples.

The wool boom was photographed and its general appearance recorded. Sampled wool knops were transported back to the laboratory and analysed within 1 hour of sampling.

Samples of the wool knops from the Lyttleton wool boom were placed into a McCartney bottle containing 5% (v/v) glutaraldehyde (BDH) (Austin, 1988) in sterile sea water. These samples were stored at 3°C until viewed using light microscopy and scanning electron microscopy (SEM).

### 2.3.2. Isolation Methods for Marine Microbes.

Microbes resident upon the wool oil boom were removed from the sampled wool knops so that they could later be isolated. Excess sea water was drained from the wool knops and 40 g wet weight of wool was weighed and placed into a sterile plastic bag. 360 ml of sterile sea water was then added and the air removed from the plastic bag. The plastic bag was placed in a Colworth 400 Stomacher, and paddled for approximately 5 mins until the wool had become cleaned of silt, and microbial biomass, and the surrounding milieu had become silty. The wool knops were then separated from the liquid, and the liquid used to form a dilution series from  $10^{-1}$  to  $10^{-9}$ , using sterile sea water as a diluent.

#### 2.3.2.1. Bacteria.

The dilutions  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$  were plated in triplicate on Marine Agar 2216 (MA) (Difco), using a pour plate technique. The plates were then incubated at  $18^{\circ}\text{C}$  for 7 days, until colonies had appeared upon the plates.

#### 2.3.2.2. Actinomycetes.

Dilutions  $10^{-2}$  to  $10^{-4}$  prepared from the stomached wool milieu were inoculated onto M2 media (Jensen *et al.*, 1991) for the isolation of the marine Actinomycetes. M2 media comprised 1% (w/v) Starch (Difco), 0.1% (w/v) Bovine Casein (Sigma), 1.6% (w/v) Bacteriological Agar, in 75% (v/v) sterilised sea water and 25% distilled water. 75  $\mu\text{g/L}$  of filter sterilised cycloheximide (Sigma) was added after autoclaving. This media was inoculated using 1 ml aliquots and the pour plate method. These plates were incubated at  $18^{\circ}\text{C}$  for 30 days, and the plates were examined regularly for growth.

#### 2.3.2.3. Fungi.

Wool knops sampled from the wool boom were placed upon Marine Fungi Agar (MFA) (Kohlmeyer and Kohlmeyer, 1979). MFA comprised 1% (w/v) glucose (BDH), 0.01% (w/v) Yeast Extract (Difco), 1.8% (w/v) Bacteriological Agar, in aged sea water. After autoclaving 0.1% (w/v) of filter sterilised streptomycin sulfate (Sigma), and 0.1% (w/v) of filter sterilised Penicillin G (Sigma), was added to the media while molten.

These plates were incubated for 14 days at  $18^{\circ}\text{C}$ , before plates were examined for fungal growth. Once growth had occurred the fungus was isolated using a cork borer, and cultured upon MFA.

### 2.3.3. Assays for Proteolytic Activity.

Microbes isolated from the wool knops were screened for their proteolytic activity, by plating on media containing gelatin.

#### 2.3.3.1. Bacteria.

One hundred bacterial colonies were selected randomly from the MA plates, and replicate plated upon Gelatin Marine Agar (GMA), and MA, by stabbing the colonies



with a sterile toothpick and transferring the bacteria to the corresponding media. GMA was adapted from the gelatin media of Hankin and Anagnostakis (1975), and comprised MA supplemented with 8% (w/v) Gelatin (Difco). The gelatin was mixed with distilled water, and left to stand for 15-30 mins prior to combining with MA. Both the GMA and MA plates were incubated at 18°C for 7 days.

After 7 days growth on GMA, these plates were flooded with 80% (w/v) ammonium sulphate  $(\text{NH}_4)_2\text{SO}_4$  (BDH) to precipitate the gelatin within the media. The number of proteolytic colonies were counted and recorded as a percentage of those plated out.

#### **2.3.3.2. Fungi.**

The fungal isolates were screened for their proteolytic activity by culturing on Marine Fungi Gelatin Agar (MFGA). This media was adapted from the media of Hankin and Anagnostakis (1975). This media consisted of MFA supplemented with 8% (w/v) gelatin, as in MGA.

Fungal cultures were inoculated by taking a plug of fungal growth using a cork borer and placing it upon the MFGA plates. These plates were incubated for 14 days at 18°C. After this time the gelatin was precipitated out of the MFGA with  $(\text{NH}_4)_2\text{SO}_4$  using the same method used earlier for MGA.

#### **2.3.4. Assays for Keratinolytic Activity.**

Microbes isolated from the wool knops, and which showed proteolytic activity, were screened for their keratinolytic activity by plating upon a media which contained KS.

##### **2.3.4.1. Bacteria**

Bacterial colonies which had shown proteolytic activity on GMA were plated on to MKA from the replicate MA plates, by stab inoculation using sterile toothpicks. Once inoculated these plates were incubated at 18°C for 14 days. The number of bacterial colonies capable of degrading soluble keratin were counted and recorded as a percentage of the colonies screened for proteolytic activity.

Control plates were also established to confirm that non-proteolytic bacterial colonies were not capable of degrading KS. This was done by plating out 100 colonies which were non-halo formers on GMA (and hence non-proteolytic), onto MKA. These plates were incubated at 18°C for 14 days.

##### **2.3.4.2. Fungi**

Proteolytic fungal cultures were examined for keratinolytic activity by plating cultures upon MKA. These plates were inoculated in the same way as the MFGA.

These plates were incubated at 18°C for 30 days. Keratinolytic activity was screened in the same way as for bacteria.

### **2.3.5. Tentative Identification of KS Degrading Bacteria.**

Colonies which were capable of degrading KS on MKA, were transferred from the replicate MA plates and purified on MA plates, for taxonomic studies.

A number of standard microbiological tests were conducted upon these cultures. These tests were Gram Stain, catalase activity, oxidase activity, and motility tests.

#### **2.3.5.1. Assimilation Tests using Modified API 20 NE Strips.**

API 20 NE strips (Biomerieux) were adapted for use with marine bacteria (MacDonell *et al.*, 1982; Breschel and Singelton, 1992) so that the assimilation tests could be used to determine information about the metabolic activity of the isolates.

A number of modifications were required to the normal API 20 NE protocol for the tests to be used with marine bacteria. The 0.85% saline diluent that is normally employed was replaced with half strength ASW (Gauthier and Breittmayer, 1992). The media supplied with the API 20 NE strips known as AUX media was replaced with a media which was suitable for marine bacteria. This media also contained Phenol Red (BDH) as an indicator to observe changes in pH, and therefore to assist in the identification of a positive result, as the media in a cupule with microbial growth would exhibit a colour change of red to yellow, due to the production of acid products by the actively growing bacteria (Breschel and Singelton, 1992). This media was known as Marine Phenol Red Base Agar (MPRBA). This media was modified from the Phenol Red Base Agar of Atlas (1993). This media consisted of 1% (w/v) Tryptone (Difco), 0.5% (w/v) NaCl, 0.0018% (w/v) Phenol Red, 0.15% (w/v) Bacteriological Agar, in full strength ASW.

The API 20 NE strips were inoculated using standard API 20 NE protocol, and adapted media. Once inoculated these strips were incubated at 18°C and scored after 48 and 72 hours, rather than the standard incubation times of 24 and 48 hours.

#### **2.3.5.2. Transmission Electron Microscopy (TEM).**

TEM was used to determine the presence of flagella, and cellular morphology. Bacteria for TEM examination were grown on MA. While the colonies were young a loopful of bacteria was removed and mixed with distilled water. These bacteria were negatively stained using 2% (w/v) phosphotungstic acid (pH 6.7). A drop of negatively stained bacteria was placed upon a copper grid, and the excess solution reaspirated after 1 to 2 min with a strip of filter paper, and the grid allowed to dry. The bacteria upon the

grid were examined under a Jeol Transmission Electron Microscope 1200X and photographed.

#### **2.3.5.3. Fatty Acid Analysis and Identification.**

Bacteria were grown on MA, and sent to Microbial ID Inc., Newark, Delaware, USA, for identification via fatty acid analysis.

### **2.3.6. Confirmation of Wool Biodegradation.**

Marine bacteria showing the ability to degrade KS, were inoculated into liquid cultures containing woollen substrates, so that their ability to degrade wool could be determined.

#### **2.3.6.1. Keratin Azure Biodegradation.**

KS degrading bacteria, isolated from the Lyttleton wool boom were inoculated into MacCartney bottles containing 15 ml of ASW and 0.1 g of sterilised Keratin Azure.

To prepare inoculum, the marine bacteria were grown on Marine Broth 2216 (Difco) (MB) for 3 days at 18°C. The cells were sedimented by centrifuging the tube at 8600 g for 15 mins in a Sigma 2K15 centrifuge. After centrifuging the supernatant was decanted and the pellet resuspended in 10 ml of ASW, to wash the cells and remove nutrients. This solution was once again centrifuged, and the pellet washed twice more. After washing, the cells were resuspended in 10 ml of ASW, so that there were  $10^7$  CFU/ml. Four ml of this solution was used to inoculate the MacCartney bottle containing 0.1 g of Keratin Azure. A sterile control was produced by using 20 ml of ASW and 0.1g of Keratin Azure in a MacCartney bottle.

The MacCartney bottles were incubated at 18°C, in a Gallencamp orbital incubator set at 150 rev/min. These tubes were examined periodically for dye release and wool fibre damage.

After 5 months incubation, the purity of the bacterial culture in the MacCartney bottle was checked by vortexing for 2 mins. One ml of the solution was diluted in a dilution series using ASW as a diluent, from  $10^{-1}$  to  $10^{-8}$ . Dilutions  $10^{-6}$ ,  $10^{-7}$ , and  $10^{-8}$  were plated out onto MA in triplicate using the pour plate method. The inoculated plates were incubated at 18°C for 7 days, until colonies had appeared.

#### **2.3.6.2. Wool Biodegradation.**

Bacteria which had shown the ability to degrade soluble keratin were inoculated into 250 ml conical flasks containing 200 ml of ASW and 0.3g of wool knops. Inoculum for the flasks consisted of  $10^7$  CFU/ml. These cultures were incubated at 18°C in a

Gallencamp orbital incubator at 150 rev/min. Wool fibres were removed at monthly intervals and observed microscopically for indications of microbial growth and damage.

After 5 months incubation, the wool knops were removed from the solution and placed in a sterile MacCartney bottle containing ASW. This tube was vortexed for 5 mins to remove bacteria attached to the wool fibres. The resulting microbial milieu was diluted, and diluted samples plated as in the method used previously for Keratin Azure degradation in 2.3.6.1..

## **2.4. MICROSCOPIC EXAMINATION OF WOOL FIBRES.**

### **2.4.1. Light Microscopy.**

Wool fibres were stained with Phenol Aniline Blue (PAB) (Austin, 1988), to highlight microbial growth and damage. This stain consisted of 0.05% (w/v) phenol (Sigma), 0.05% (w/v) water soluble Aniline blue (BDH), in 20% (v/v) acetic acid. The wool fibre was placed in PAB for 30 seconds, removed and transferred to distilled water for 30 seconds. Once stained the wool fibre was wet mounted in distilled water upon a microscope slide and a coverslip placed on top of the wool fibre, and sealed using nail polish, to prevent desiccation of the sample.

Stained fibres were observed and photographed using an Olympus BH-2 Light Microscope.

### **2.4.2. Scanning Electron Microscopy.**

SEM was conducted upon wool samples which had been fixed and stored in 5% (v/v) glutaraldehyde. Wool knops were removed from the glutaraldehyde and dehydrated by passing through an ethanol series (30%, 50%, 70%, 90%, 95%, and 100%), and then through an acetone series (30%, 50%, 70%, 80%, 90%, 100%).

Dehydrated samples were critically point dried and placed upon stubs. The wool fibres were coated with gold for 5 mins, and photographed using a Cambridge Sterocam 250 mk2 Scanning Electron Microscope.

## **2.5. EFFECT OF OIL ON WOOL DEGRADATION.**

Cultures containing oil absorbed onto wool knops, were established for microbes in sea water, and keratinolytic bacteria, so that wool degradation could be monitored in the laboratory, by light microscopy, and the release of free amino groups.

Culture flasks of keratinolytic bacteria were established in 250 ml conical flasks as previously for the wool degradation experiments. In addition however, cultures were established that contained oil adsorbed onto wool knops. Twenty ml of crude Arabian

light oil (Shell) was placed into 250 ml conical flasks. To each flask 1 g of sterile wool knops were added, and the wool knops and oil mix stirred for 15 mins, to allow adsorption of the oil onto the wool fibres. 200 ml of sea water, LBM, or SFBM, were then added to each flask. The SFBM and LBM containing cultures were then inoculated with *Lysobacter*, or *S. fradiae*, according to the methodology used previously for the Keratin Azure degradation studies in 2.2.2.1.1.. Controls were established for these flasks using the methodology used previously for wool degradation, however additional flasks were established which contained LBM or SFBM as well as oil and wool knops, and which were not inoculated.

Sea water cultures and controls were established as for keratinolytic cultures. Instead of using basal salts media, 200 ml of sea water was used as inoculum. Control flasks were established using sterile sea water, in a similar procedure to that used in the keratinolytic cultures.

The cultures were sampled periodically using the same methodology previously for wool degradation analysis in section 2.2.2.2.. The culture samples were tested for free amino acid concentrations, and single wool fibres were removed, and examined for microbial growth and fibre damage, using light microscopy.

## **2.6. EFFECT OF ABIOTIC FACTORS IN SEA WATER ON WOOL STRENGTH.**

The effect of abiotic factors in sea water, upon wool degradation was determined using wool strength testing. This was achieved by placing a piece of sterile woollen fabric (supplied by WRONZ), into a 500 ml conical flask, containing 300 ml of sterile sea water, and incubating at 18°C.

At monthly intervals the fabric was removed, and a section 3 cm x 10 cm removed from the fabric under aseptic conditions. The sections of fabric were frayed down to approximately 2 cm by 7 cm, with an equal number of yarns across the width. Wool strength tests were performed on the fabric pieces by WRONZ using an Instron 4204 Tensile Strength Tester.

### 3. RESULTS.

#### 3.1. BIODEGRADATION OF WOOL *IN VITRO*.

Wool was biodegraded by marine microbes. This was seen in during *in vitro* studies using Keratin Azure, and wool knops. The degradation of wool in these controls were compared to the degradation of these substrates by pure cultures of the keratinolytic bacteria *S. fradiae*, and *Lysobacter*.

##### 3.1.2. Biodegradation of Keratin Azure.

Keratin Azure was degraded by mixed cultures of microbes from sea water, and pure cultures of the keratinolytic bacteria, *S. fradiae* and *Lysobacter*. Degradation was indicated by stain liberation from the Keratin Azure (Figure 6), and microscopic examination of the Keratin Azure fibres.

Mixed cultures of bacteria enriched from sea water were unable to degrade Keratin Azure as rapidly as the keratinolytic bacteria (Figure 6). Stain liberation for the bacteria enriched from sea water did not begin until 8 weeks, and after 32 weeks the amount of stain liberated by the culture was less than that which was released by the pure cultures of keratinolytic bacteria after 28 days.

Microscopic examination of the Keratin Azure fibres in the mixed cultures of marine microbes, showed that pitting had occurred in the fibres after 12 weeks (Figure 7). This indicated that degradation of the fibre had begun. As stain release increased so did the corresponding amount of damage. Microbial growth upon the fibres was difficult to observe due to the blue colouration of the fibres. After 32 weeks the fibres were not entirely degraded, although the length of the remaining fragments were much shorter than those originally used. Large pits were seen over much of the fibres.

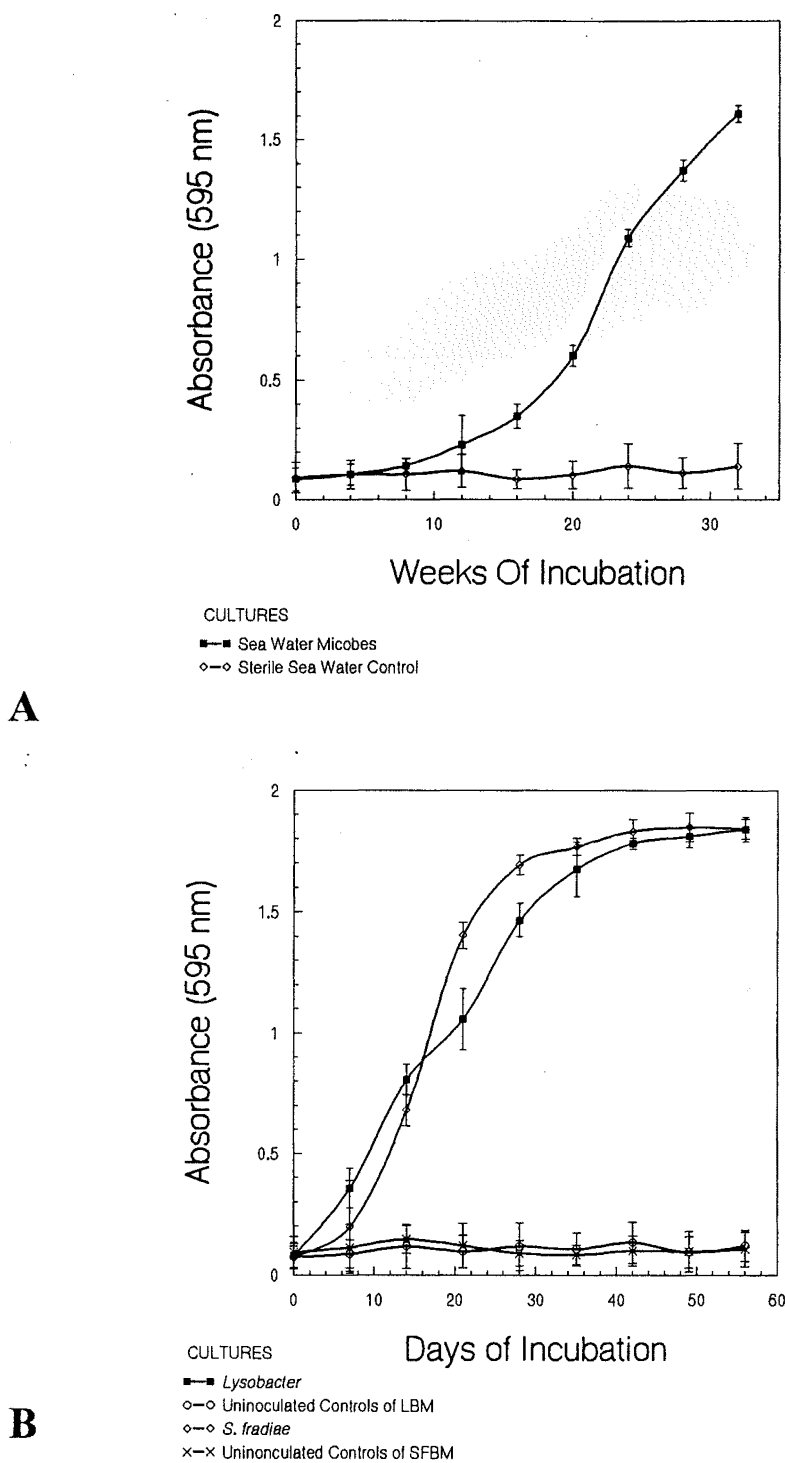
*S. fradiae* and *Lysobacter* rapidly degraded Keratin Azure, as measured by stain liberation. Both bacteria were able to degrade Keratin Azure to the same extent.

Microscopic examination of the Keratin Azure fibres revealed that the fibres were degraded by first attacking the cuticle of the fibre, and then releasing cortical cells into the culture solutions. When maximum stain liberation was achieved the culture solutions no longer contained intact wool fibres, and only free cortical cells were visible.

Uninoculated control cultures showed no stain release or fibre damage.

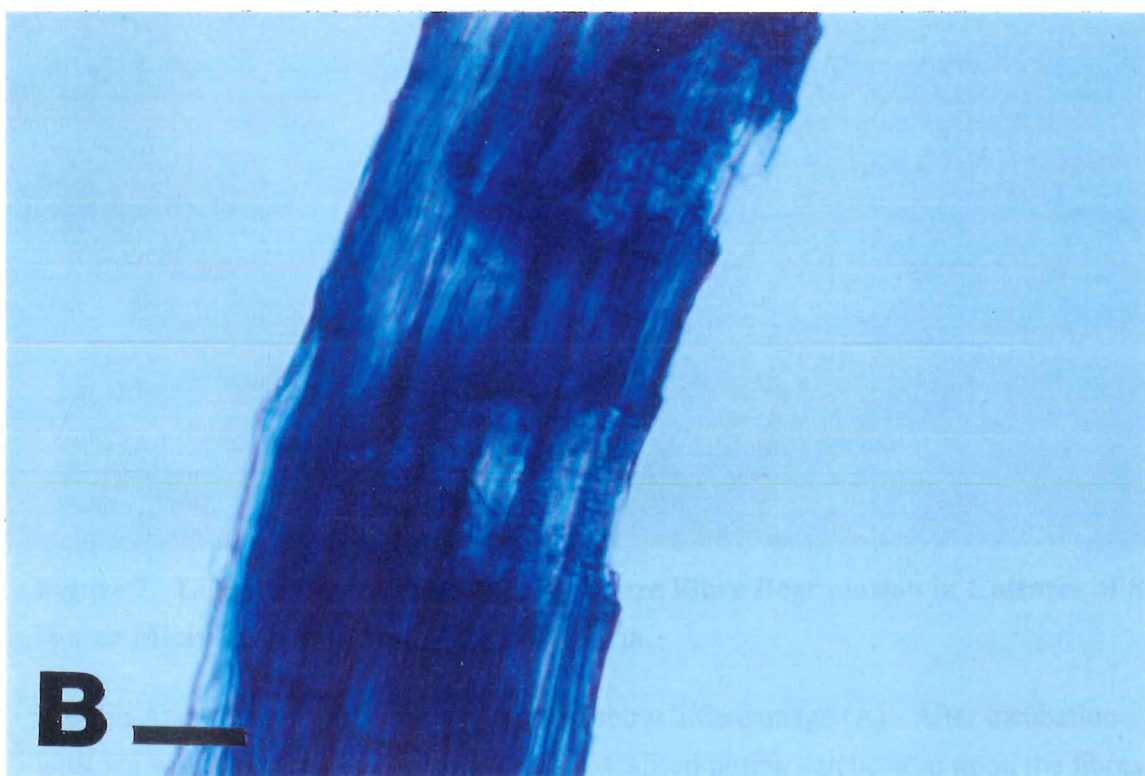
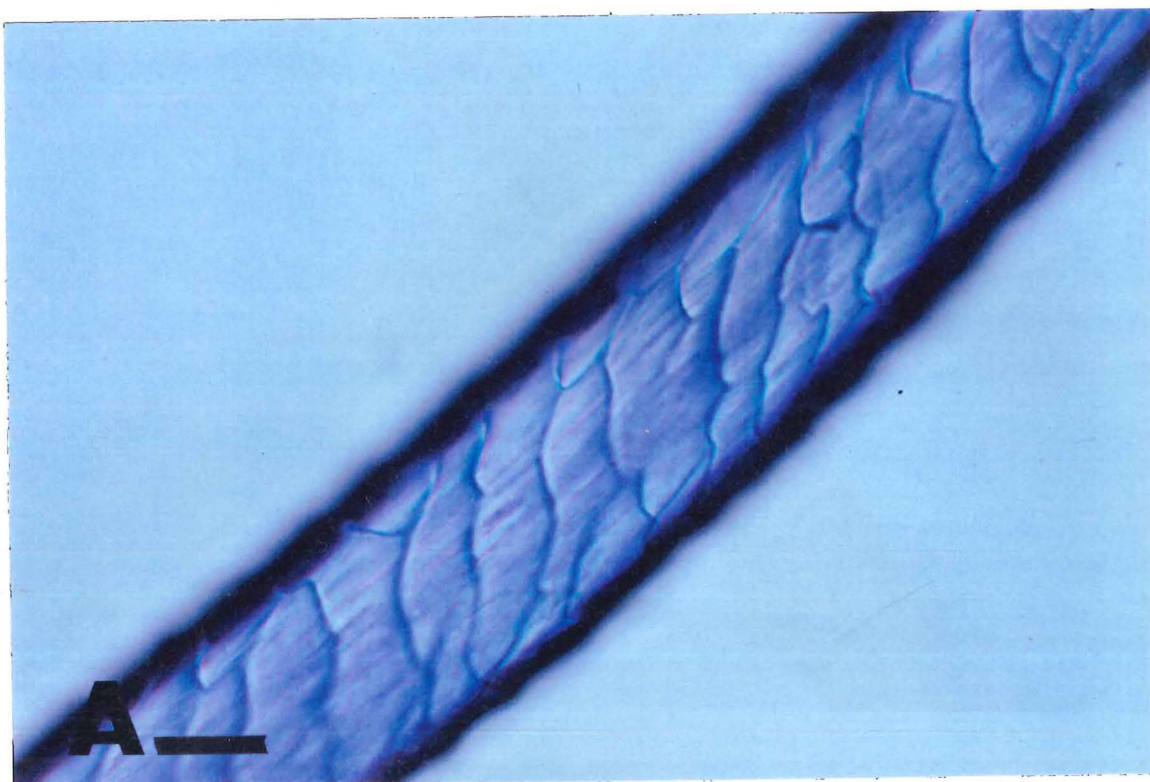
##### 3.1.2. Biodegradation of Wool.

Wool knops were degraded by mixed cultures of marine microbes enriched from sea water, and by pure cultures of the keratinolytic bacteria, *S. fradiae* and *Lysobacter*.

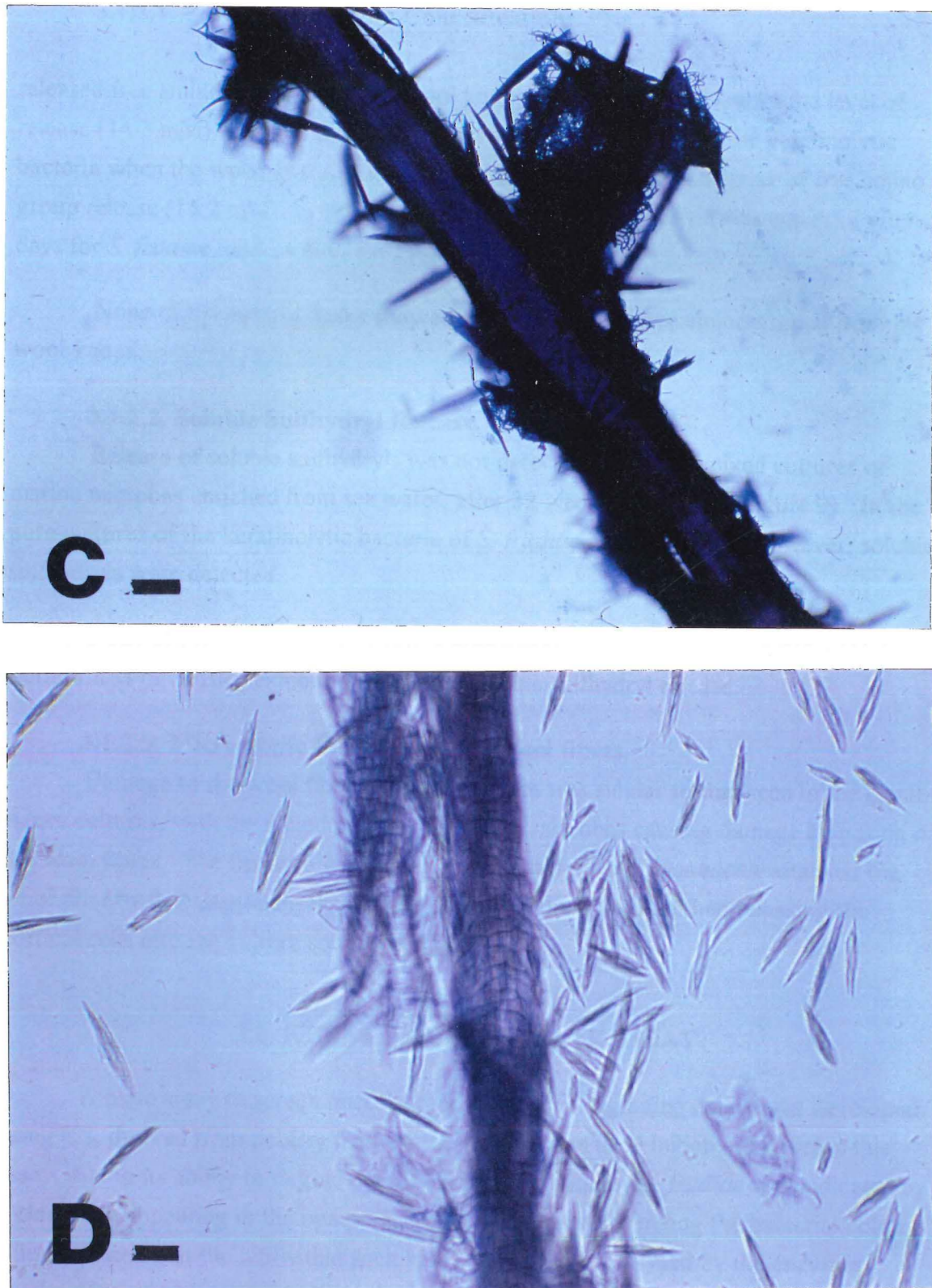


**Figure 6. Stain Liberation from Keratin Azure by Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

Keratin Azure degradation was monitored in cultures containing sea water microbes (A), and the keratinolytic bacteria *S. fradiae* and *Lysobacter* (B), by the liberation of blue stain from the woollen substrate. From these results it is apparent that the degradation of Keratin Azure was much slower in mixed cultures of marine microbes, than pure cultures of the keratinolytic bacteria.







**Figure 7. Light Microscopy of Keratin Azure Fibre Degradation in Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

Keratin Azure fibres which are undegraded show little damage (A). After incubation with sea water microbes for 28 weeks (B), localised pitting can be seen upon the fibres. Incubation with *S. fradiae* (C), and *Lysobacter* (D) for 21 days, causes cortical cells to be removed from the fibres (bar = 10  $\mu$ m).

### 3.1.2.1. Free Amino Group Concentrations.

released free amino groups from the wool knops slowly. After 32 weeks the level of release (14.3 mM) was slightly less than that seen in the cultures of the keratinolytic bacteria when the wool, in these cultures had been solubilised. The peak of free amino group release (15.2 mM) for both cultures of keratinolytic bacteria was achieved after 21 days for *S. fradiae*, and 28 days for *Lysobacter*.

None of the control flasks showed any liberation of free amino groups from the wool knops.

### 3.1.2.2. Soluble Sulphydryl Release.

Release of soluble sulphydryls was not detected within the mixed cultures of marine microbes enriched from sea water, after 32 weeks incubation (Figure 9). In the pure cultures of the keratinolytic bacteria of *S. fradiae* and *Lysobacter* however, soluble sulphydryls were detected.

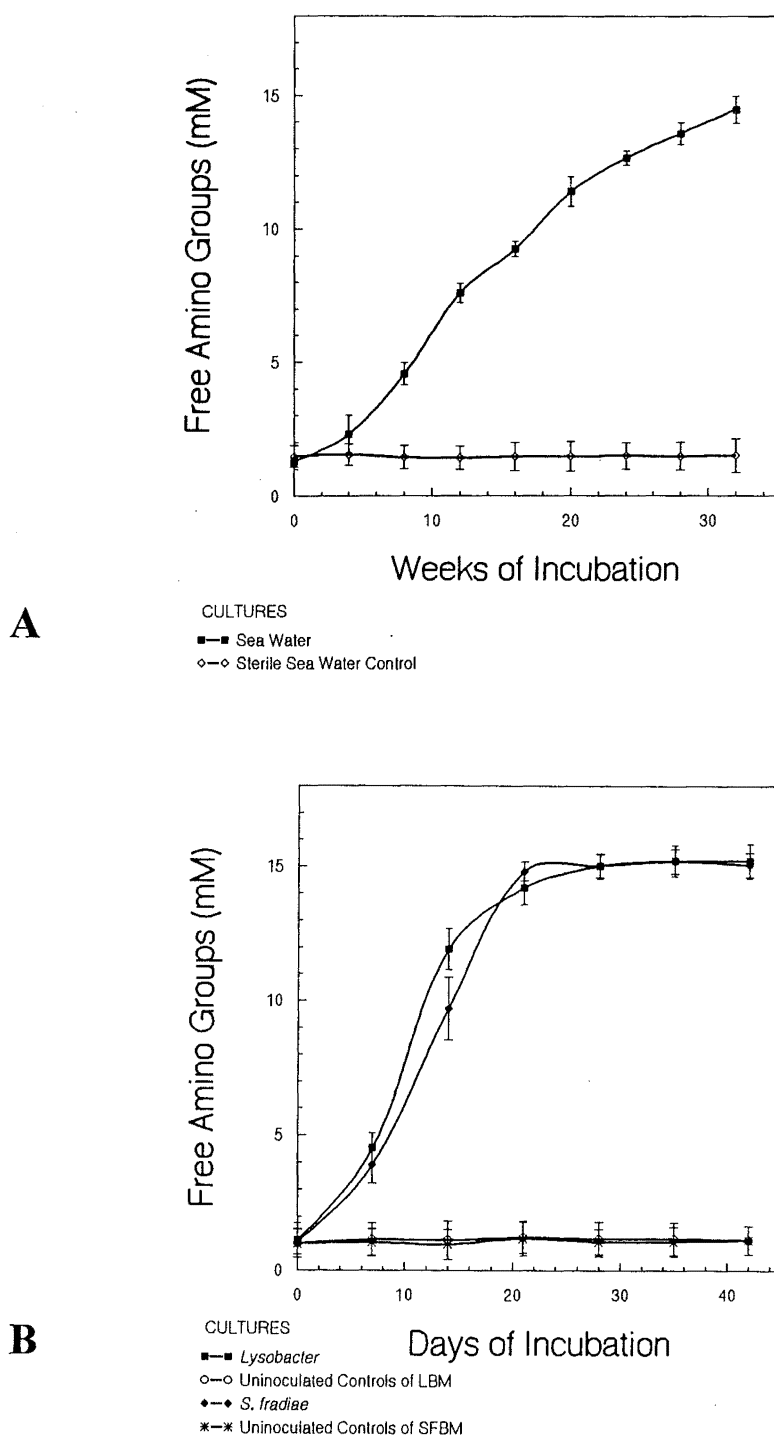
None of the uninoculated controls containing wool in sterile sea water, or wool in sterile LBM or SFBM, exhibited detectable soluble sulphydryl release.

### 3.1.2.3. Microscopic Examination of Wool fibres.

Damage to the wool fibres in these cultures was similar to that seen in the Keratin Azure cultures, with the mixed cultures of marine microbes causing damage by pitting of the wool fibres. The keratinolytic bacteria of *S. fradiae*, and *Lysobacter* attacked the wool fibre by first degrading the cuticle (scales) of the fibre, and then releasing the cortical cells into the culture solution (Figure 10).

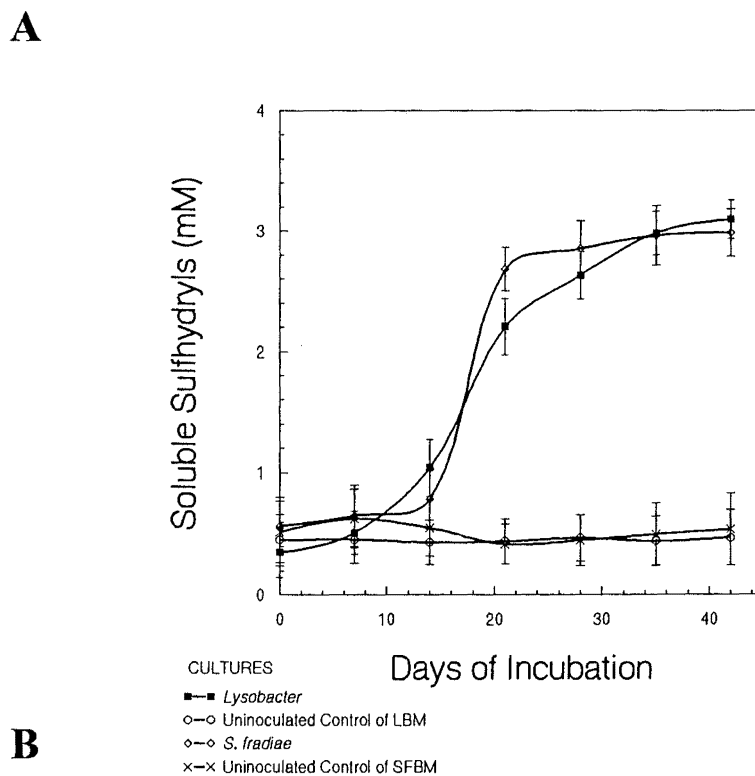
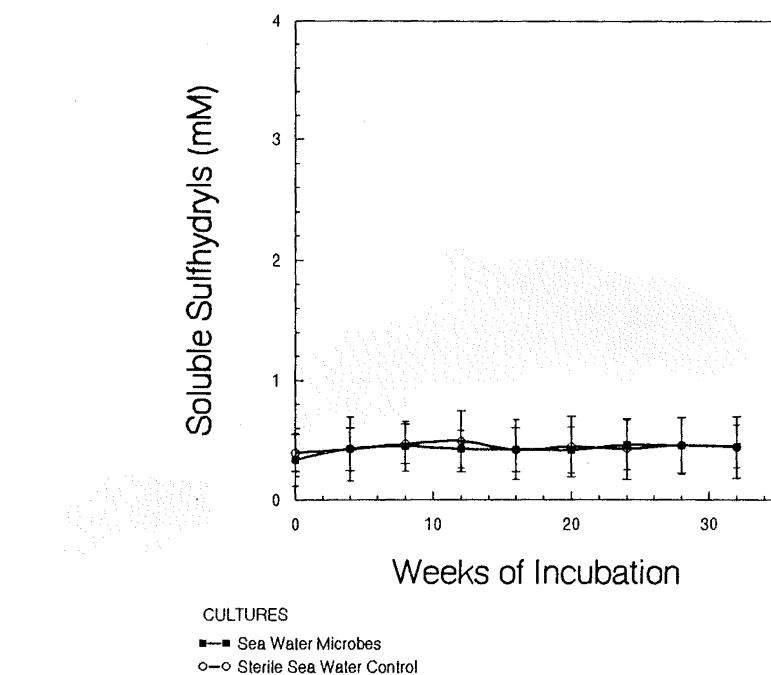
## 3.2. KERATINOLYTIC PLATE ASSAY.

A plate assay to screen microbes for their wool degrading ability was developed using KS, derived from poultry feathers. *S. fradiae* was used initially to develop this assay, due to its ability to degrade wool. KS degradation by *S. fradiae* was indicated by a clear halo appearing in the opaque KS layer, in areas surrounding the bacterial colony. This indicated that the KS within such haloes had been hydrolysed by the enzymes produced by the bacteria within these colonies. By placing KS in a media containing ASW, a similar assay was developed for screening marine isolates. Degradation of KS by marine bacteria, isolated during preliminary studies, appeared similar to that seen for KS degradation by *S. fradiae* (Figure 11). This indicated that KS could be used in a plate assay to rapidly screen marine microbes for their wool degrading ability.



**Figure 8. Free Amino Group Release During Wool Knop Degradation by Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

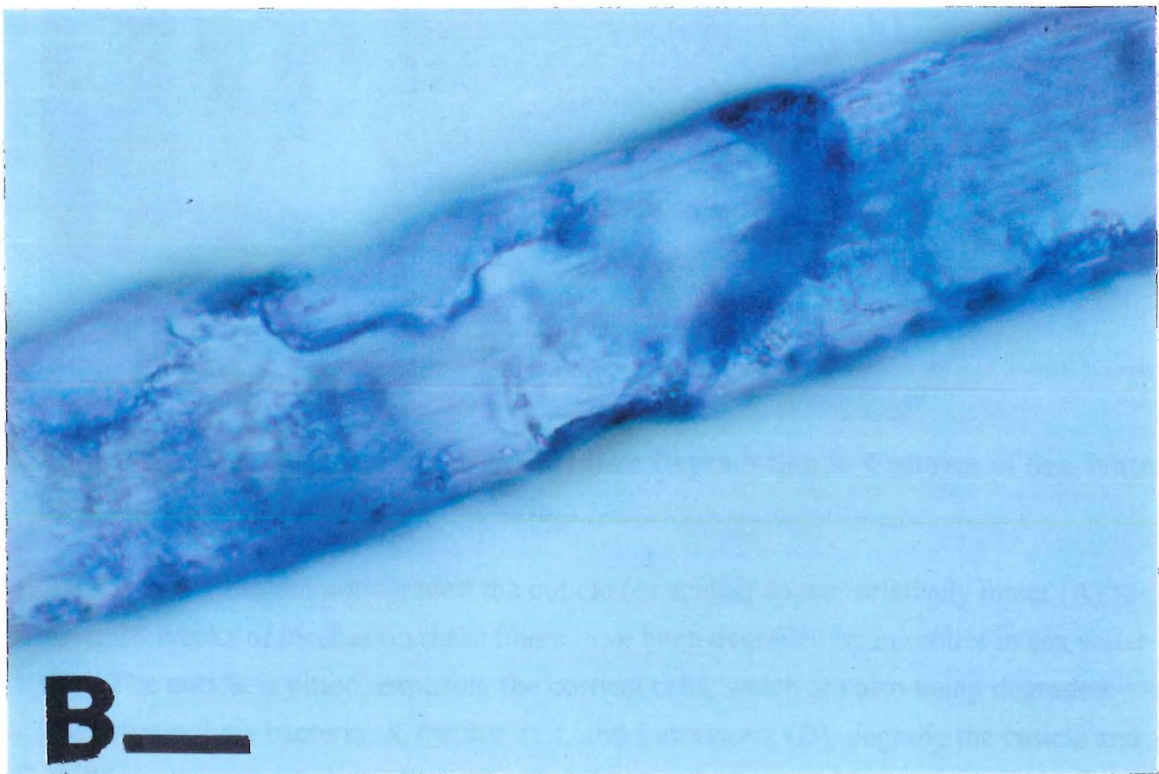
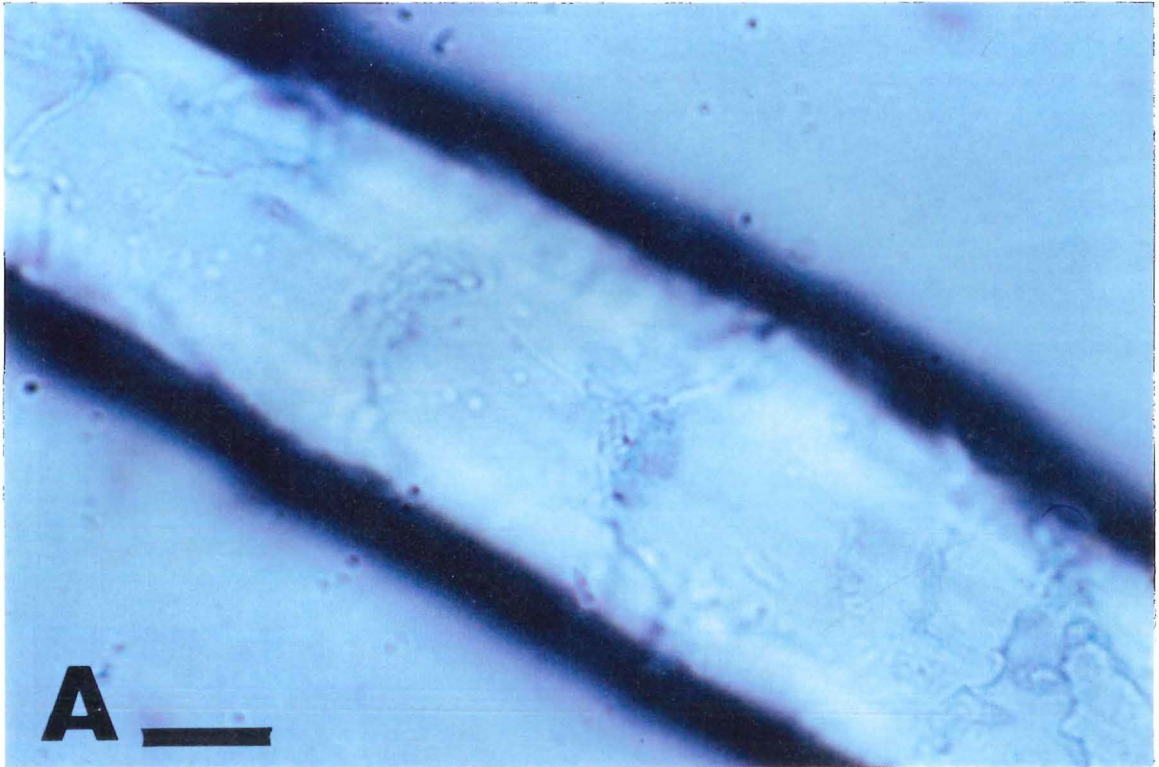
Free amino groups were released during the degradation of wool knops by marine microbes in sea water (A), and the keratinolytic bacteria *S. fradiae*, and *Lysobacter* (B). Free amino group release was much slower in the culture containing sea water microbes, than in the keratinolytic bacterial cultures.

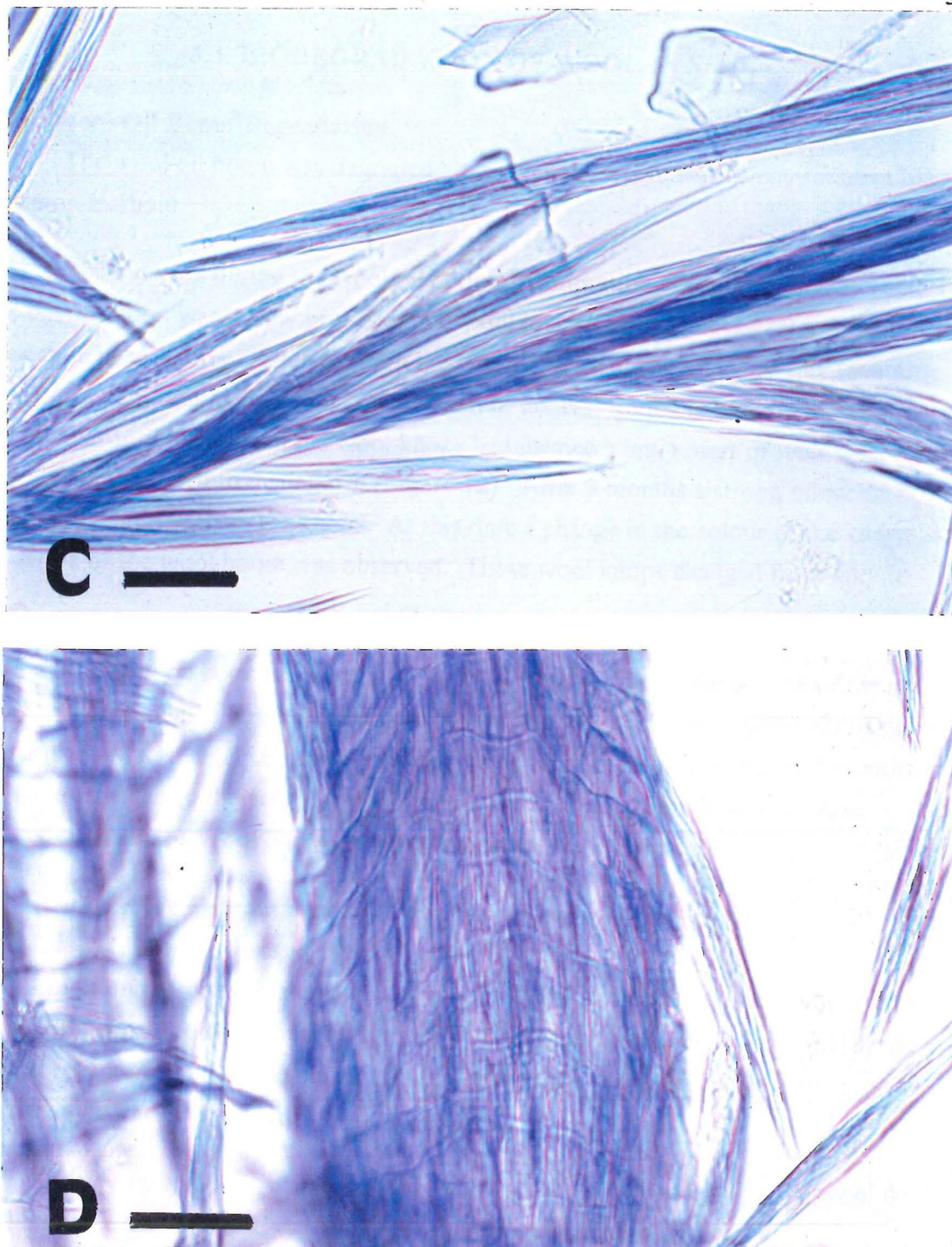


**Figure 9. Soluble Sulfhydryl Release During Wool Knop Degradation in Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

Soluble sulfhydryls were not detected during the degradation of wool knops by marine microbes in sea water (A). They were however, detected within the culture media of *S. fradiae*, and *Lysobacter* (B).







**Figure 10. Light Microscopy of Wool Fibre Degradation in Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

When wool fibres are undegraded the cuticle (or scales) appear relatively intact (A). After 28 weeks of incubation these fibres have been degraded by microbes in sea water (B). The cuticle is pitted, exposing the cortical cells, which are also being degraded. The keratinolytic bacteria, *S. fradiae* (C), and *Lysobacter* (D), degrade the cuticle and the cement surrounding the cortical cells releasing them from the wool fibre. All fibres are stained with PAB to highlight microbial growth and wool fibre damage (bar = 10  $\mu\text{m}$ ).



### 3.3. BIODEGRADATION OF WOOL *IN SITU*.

#### 3.3.1. Wool Oil Boom Degradation.

The wool oil boom was degraded after exposure to the marine environment of Lyttleton Harbour.

Observations during the wool oil booms deterioration are summarised in Table 1. The deterioration was a slow process, total wool loss was not achieved after 8 months immersion in sea water. After 1 month the wool knops had become very silty, and grey in colour. At 3 months the wool knops began to unravel from a ball shape, while at 8 months the wool fibres from the wool knops had formed a large mass of wool fibres with individual knops hard to distinguish (Figure 12). After 3 months a strong odour of H<sub>2</sub>S began to be emitted from the knops. At this time a change in the colour of the knops in the centre of the wool boom was observed. These wool knops changed from grey to black.

The wool oil boom was originally buoyant, floating upon the surface of the sea water. After 2 months however, the wool boom had sunk and was suspended just below the surface. Macro algal communities began to appear upon the boom after 5 months, and were attached to the polypropylene mesh. By 8 months the whole boom was covered with algae. Wool boom deterioration is shown in Figure 13.

#### 3.3.2. Microscopic Examination of Wool Fibre Degradation.

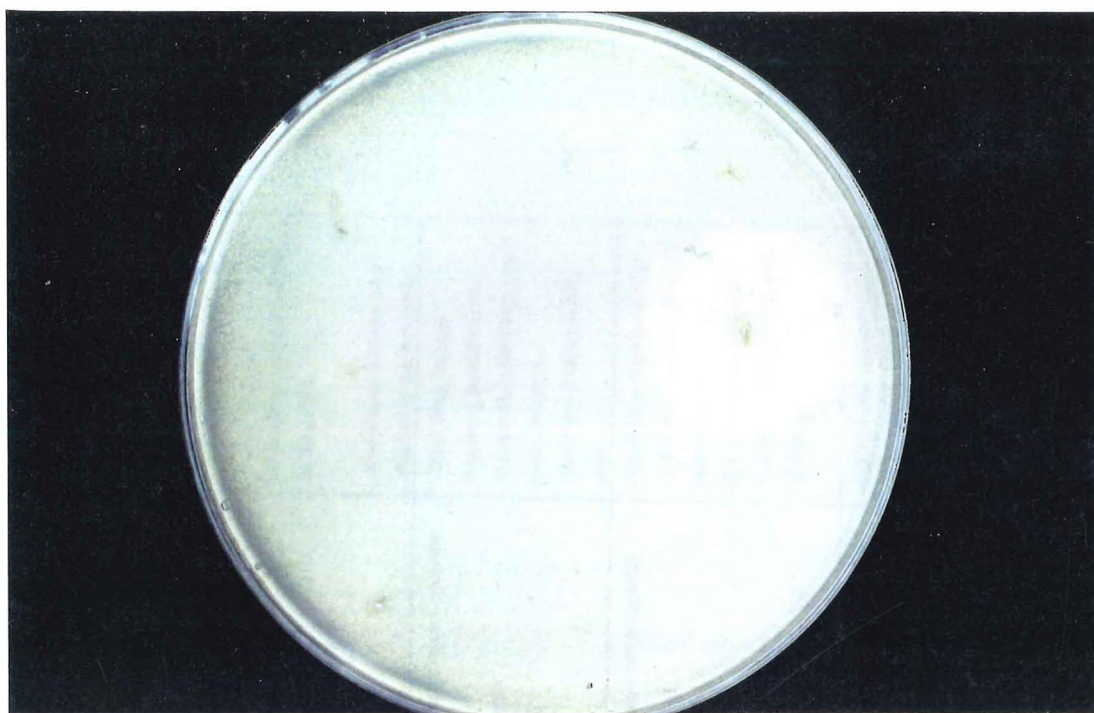
##### 3.3.2.1. Light Microscopy.

Light microscopy to show microbial growth on, and damage to the wool fibres, was greatly enhanced by the use of the stain PAB. PAB stained microbial growth, and wool fibre damage blue. Microbial growth, and damage, as observed by light microscopy is shown in Figure 14.

After 1 month no damage was observed, and microbial growth on the wool fibre was limited. Small bacterial colonies were occasionally observed.

After 2 and 3 months, still no wool damage was observed, however microbial growth upon the wool fibres had increased, and bacterial colonies appeared considerably larger than after 1 month.

After 4 and 5 months, small, shallow, and infrequent pits appeared upon the wool fibre. These seemed to penetrate the cuticle. Microbial growth on the wool fibre had become more extensive and covered most of the fibre, with individual bacterial colonies hard to distinguish. At 4 months, chains of algal cells were also occasionally seen looping around the wool fibre.



**Figure 11. Keratinolytic Plate Assay.**

Keratinolytic activity in this media was indicated by the formation of a clear halo within the opaque KS layer, surrounding the marine bacterial colony.



**Figure 12. Unravelling of Wool Knops during Wool Degradation in Lyttleton Harbour.**

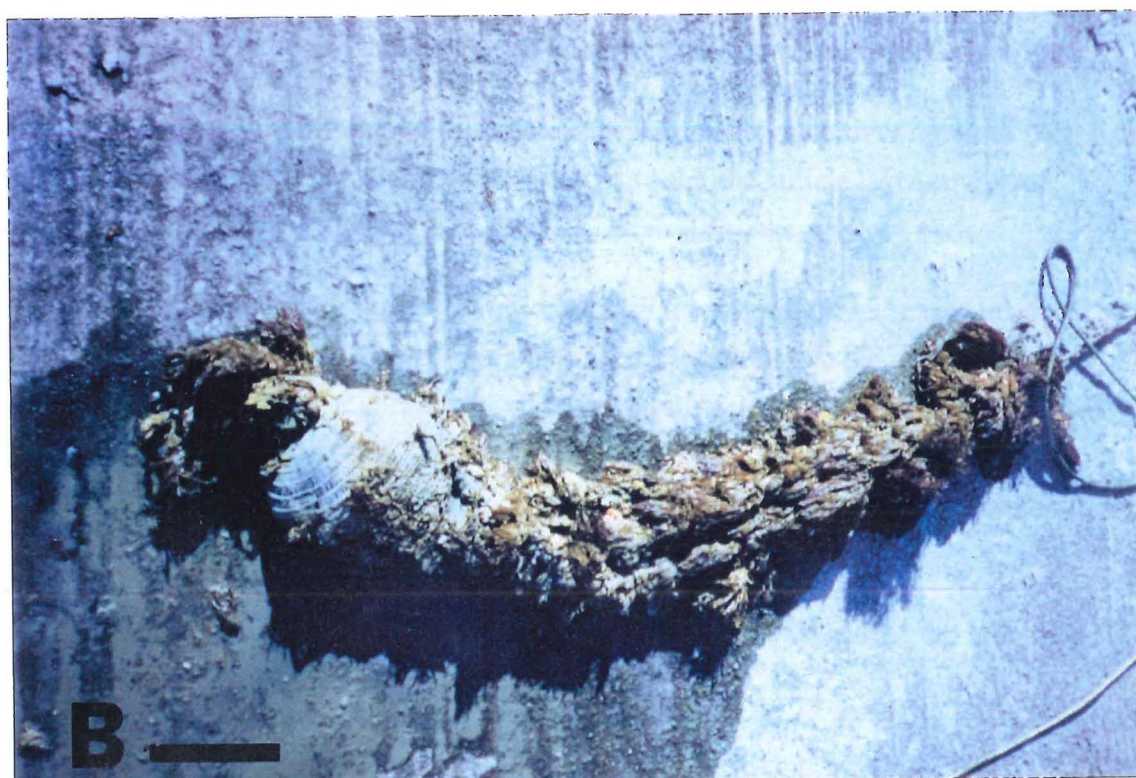
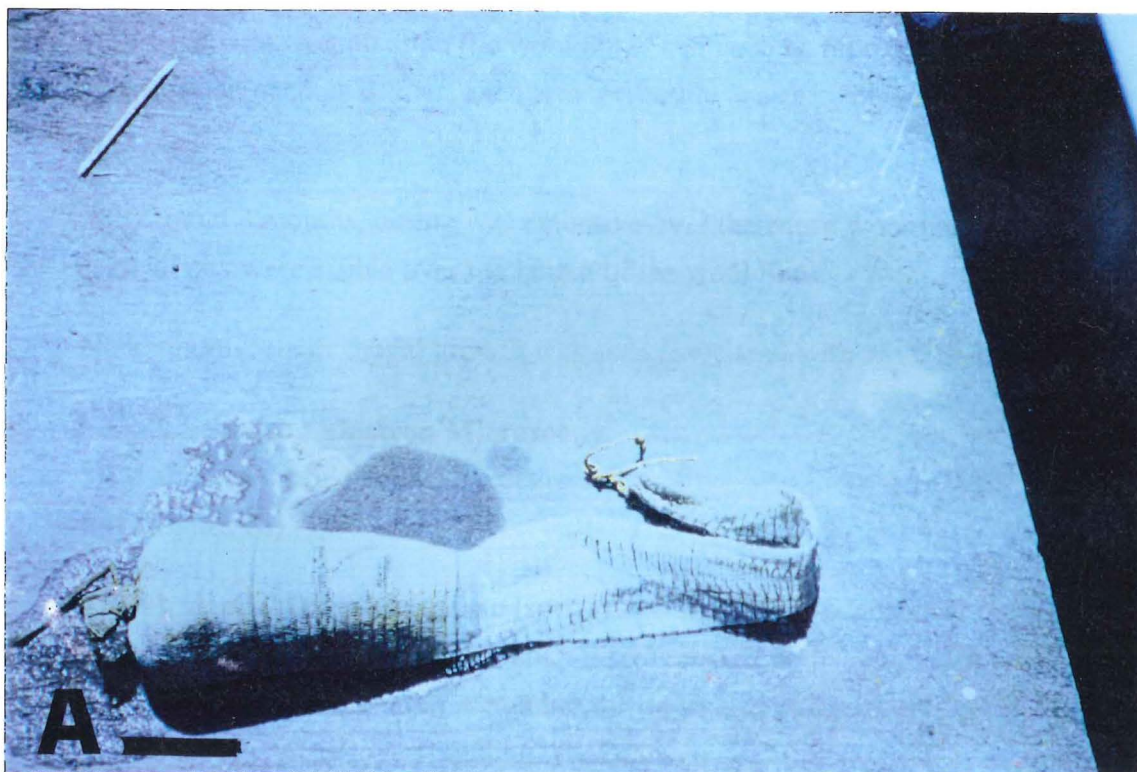
Degradation of the wool fibres from the wool oil boom in Lyttleton Harbour, caused the wool knops to unravel. This can be seen at 0, 2, 4, 6, and 8 months.



MONTH	OBSERVATIONS		
	Wool Boom	Light Microscopy	SEM
1	<ul style="list-style-type: none"> <li>-Area of wool knops in the boom slightly reduced</li> <li>-Slight discolouristion of the wool knops</li> <li>-Knops still tightly held in a ball shape</li> </ul>	<ul style="list-style-type: none"> <li>-Microbial growth limited</li> <li>-Occasional bacterial colony</li> <li>-No wool fibre damage</li> </ul>	<ul style="list-style-type: none"> <li>-Wool fibre damage rare</li> <li>-Occasional scale damage or lifting</li> <li>-Occasional holes appearing in the cuticle</li> <li>-Rod shaped bacteria seen attached to the wool fibre</li> </ul>
2	<ul style="list-style-type: none"> <li>-Area of wool knops severely reduced</li> <li>-Wool knops very silty - grey in colour</li> <li>-Knops still tightly ball shaped</li> </ul>	<ul style="list-style-type: none"> <li>-Microbial growth more frequent upon the fibre</li> <li>-Bacterial colonies larger</li> <li>-No wool fibre damage</li> </ul>	<ul style="list-style-type: none"> <li>-Wool fibre damage becoming more frequent</li> <li>-holes with in the fibre cuticle more apparent.</li> <li>-Rod shaped bacteria still seen</li> </ul>
3	<ul style="list-style-type: none"> <li>-Area of wool knops in the boom slightly less than previous month</li> <li>-Strong H<sub>2</sub>S odour originating from the boom</li> <li>-Knops in the centre of boom turning black</li> <li>-Fibres in knops beginning to unravel</li> </ul>	<ul style="list-style-type: none"> <li>-Microbial growth almost covering total wool fibre</li> <li>-No wool fibre damage</li> </ul>	<ul style="list-style-type: none"> <li>-Wool fibre damage similar to that of 2 months, although more frequent</li> <li>-Rod shaped bacteria still observable, also seen in areas of damage</li> </ul>
4	<ul style="list-style-type: none"> <li>-Area of wool knops in the boom slightly decreased</li> <li>-Strong H<sub>2</sub>S odour still apparent</li> <li>-Polypropylene mesh very slimy</li> <li>-All wool knops becoming darker</li> <li>-Knops beginning to lose their ball shape</li> </ul>	<ul style="list-style-type: none"> <li>-Microbial growth forming a thick biofilm over the fibres</li> <li>-Individual bacterial colonies indistinguishable</li> <li>-Chains of algal cells seen looping around wool fibres</li> <li>-Wool fibre damage seen as shallow infrequent pits</li> </ul>	<ul style="list-style-type: none"> <li>-Pitting in the cuticle layer larger, deeper ,and more frequent</li> <li>-Rod shaped bacteria still observable</li> </ul>

5	<ul style="list-style-type: none"> <li>-Area of wool knops similar to previous month</li> <li>-Strong H<sub>2</sub>S odour</li> <li>-Some macro-algae attached to the polypropylene mesh</li> <li>-knops similar to previous month</li> </ul>	<ul style="list-style-type: none"> <li>-Biofilm thick</li> <li>-Pitting more frequent</li> </ul>	<ul style="list-style-type: none"> <li>-Pitting is deeper than previous months, with the exposure of the cortical cells in the fibre</li> </ul>
6	<ul style="list-style-type: none"> <li>-Wool oil boom in similar condition to previous month</li> <li>-Macro-algae coverage increasing</li> <li>-Small worm-like animals found in wool knops</li> <li>-Knops very loose although individual knops are still distinguishable</li> </ul>	<ul style="list-style-type: none"> <li>-Biofilm thick</li> <li>-Pits enlarging in depth and width</li> </ul>	<ul style="list-style-type: none"> <li>Pitting similar to previous month</li> </ul>
7	<ul style="list-style-type: none"> <li>-Wool boom in similar condition to previous months</li> <li>-Macro-algae totally covering boom</li> <li>-Knops in similar state as previous month</li> </ul>	<ul style="list-style-type: none"> <li>-Biofilm thick</li> <li>-Pits of different sizes over entire fibre surfaces.</li> </ul>	<ul style="list-style-type: none"> <li>-Pitting of different sizes spread over entire wool fibre</li> <li>-Some pits extremely deep, as the cortical cells have been degraded</li> </ul>
8	<ul style="list-style-type: none"> <li>-Area of wool knops in boom small</li> <li>-Macro-algae covering boom</li> <li>-Knops are a mass of fibres with individual knops hard to distinguish</li> <li>-Strong H<sub>2</sub>S odour</li> <li>-Knops very dark in colour</li> </ul>	<ul style="list-style-type: none"> <li>-Biofilm and pitting similar to previous month</li> </ul>	<ul style="list-style-type: none"> <li>-Pitting similar to previous month</li> <li>-Rod shaped bacteria observable upon the wool fibres and also seen in surrounding areas of pitting</li> </ul>

**Table 1. Observation of Wool Oil Boom Deterioration in Lyttleton Harbour.**



**Figure 13. Wool Oil Boom Deterioration.**

As the wool fibres within the wool oil boom were degraded, the woollen component of the boom decreased in size, as can be seen at 5 months (A). At 8 months, the wool oil boom was covered with macro-algae, which had attached themselves to the polypropylene mesh (B) (bar = 10 cm).

After 6 months, pitting upon the wool fibres had become more prevalent. Some of the older pits had become deeper, and were increasing in size. The biofilm remained extensive.

After 7 and 8 months, pitting was extensive over the entire wool fibre. Both large and small pits were visible over the length of the wool fibre.

No actinomycete or fungal growth was seen associated with wool fibres.

### **3.3.2.2. Scanning Electron Microscopy.**

SEM showed more detail of the degradation of the wool fibre than light microscopy (Figure 15).

After 1 month, damage to cuticle (scales) was seen. This occurred in the form of scale breakdown and scale lifting. Small holes also appeared in the cuticle, although this type of damage was extremely rare. A number of rod shaped bacteria were seen on the wool fibres, especially in areas surrounding wool damage.

After 2 to 3 months, the wool fibre damage was similar to that which had been seen after 1 month, although the amount of damage to the fibre had increased.

After 4 to 5 months, pits of different sizes were observable over much of the wool fibres. Pitting appeared to have penetrated through the cuticle layer, and had exposed the cortical fibres for degradation.

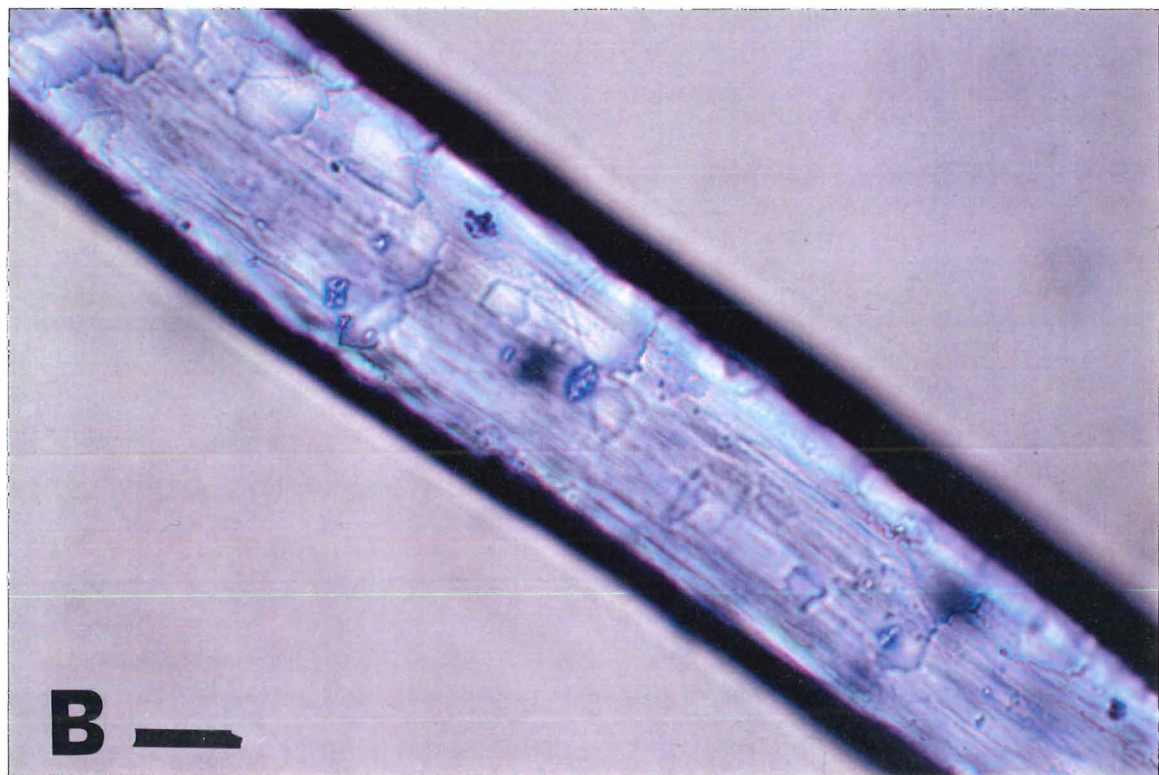
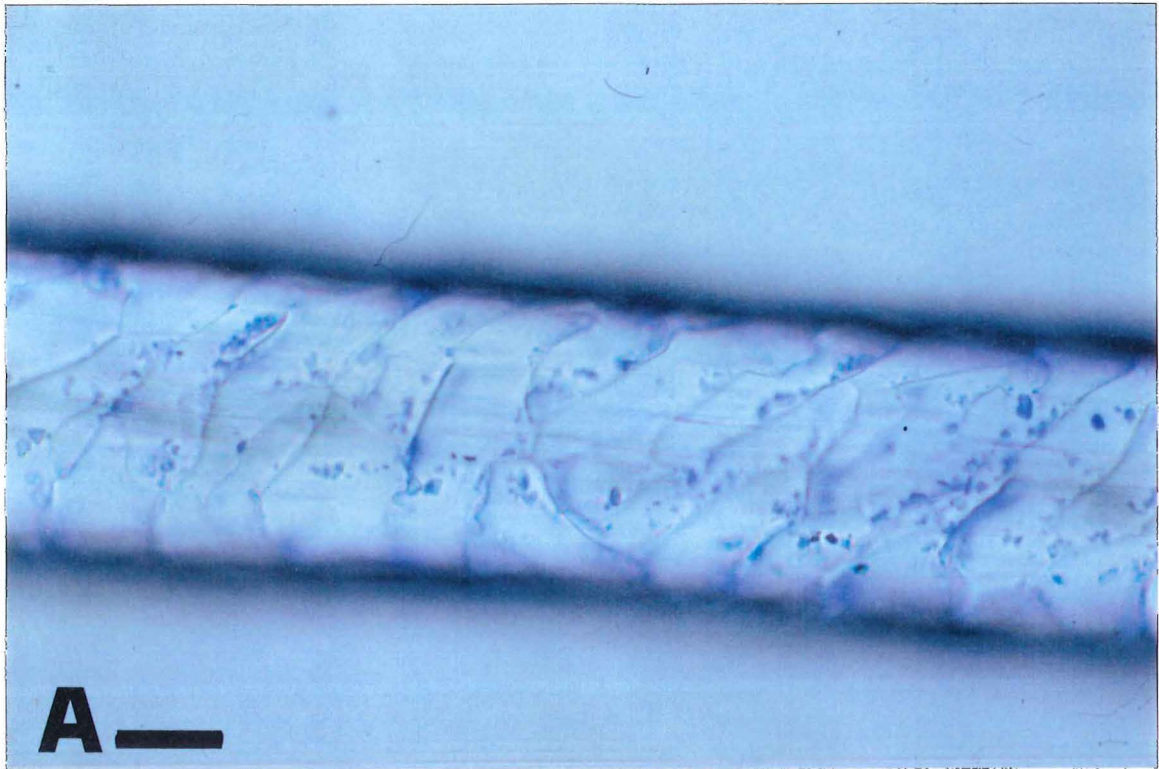
After 6, 7, and 8 months, the deterioration in the wool fibre increased, as did the depth of the pits. The cortical cells which had been exposed in the pitting had begun to be removed and therefore the pits appeared to penetrate deep into the wool fibre. Other smaller pits were also noticeable, clustered in areas of intact fibre, indicating that the process of pitting was beginning in these regions. Rod shaped bacteria were regularly seen associated with the wool fibres, and in areas of wool damage.

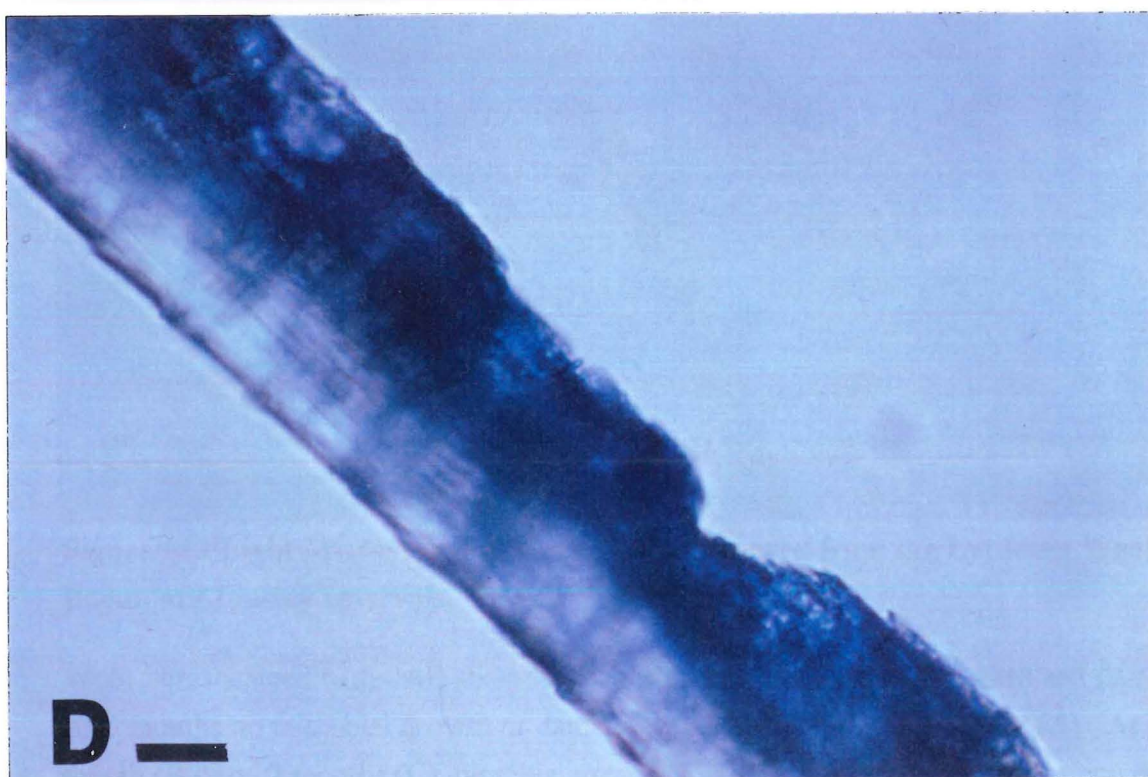
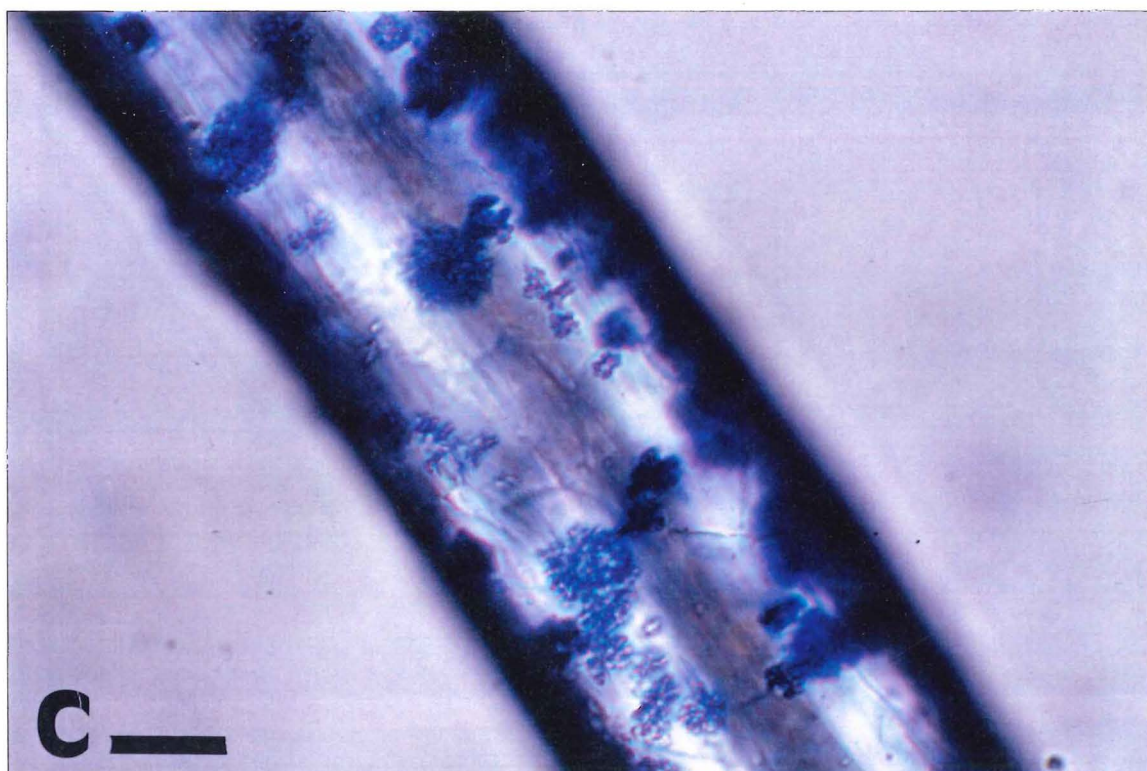
Throughout the observation period, of the fibre samples by SEM, no fungal or actinomycete growth was observed.

### **3.3.3. Isolation of Microbes.**

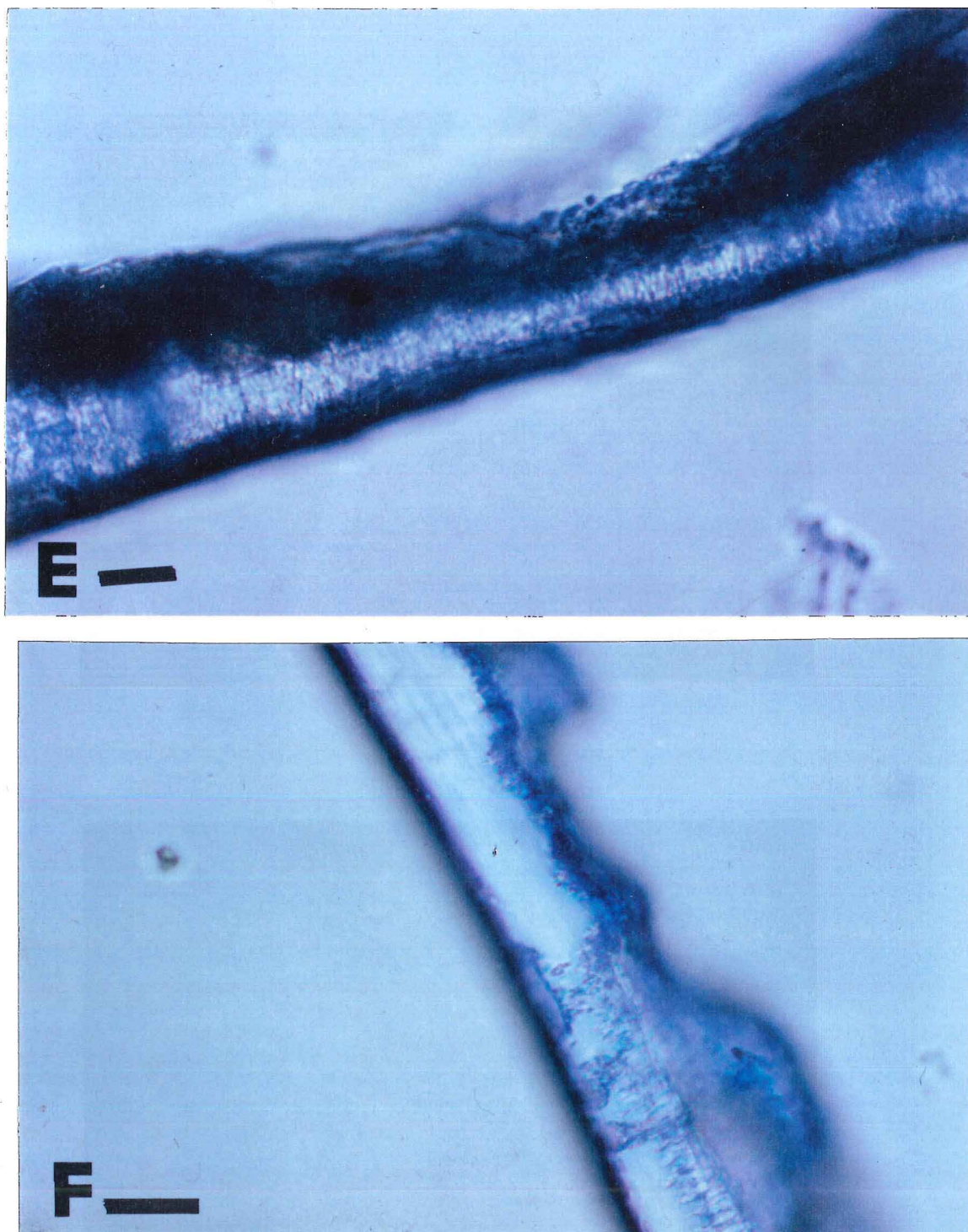
The use of a stomacher in removing microorganisms attached to wool boom fibres, proved successful. Examination of wool fibres after stomaching by light microscopy, showed that most of the attached biofilm, and microbes had been removed. After the dilution of the liquid from the stomached wool, and plating onto MA, a number of microorganisms were isolated.





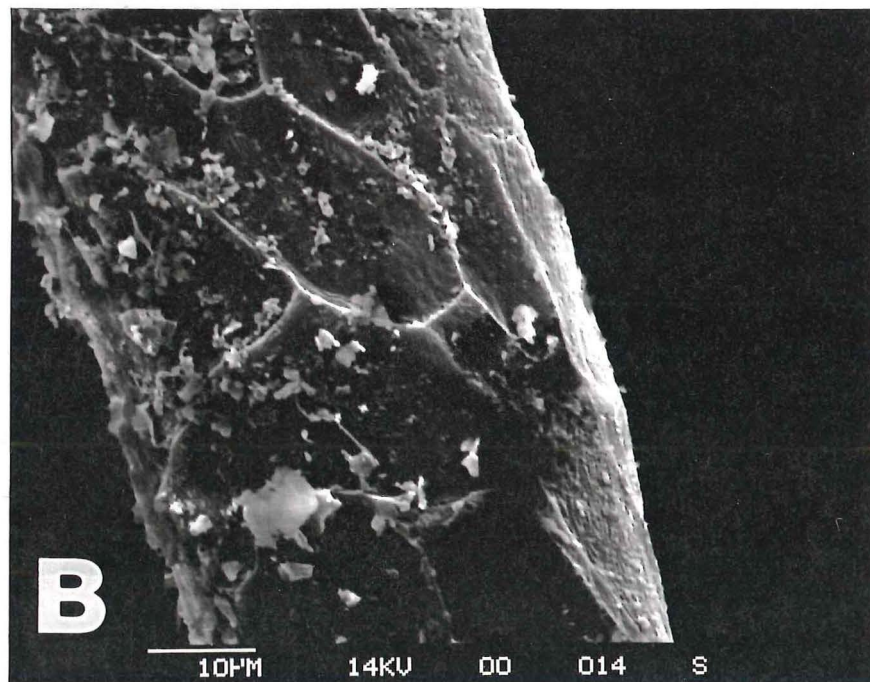
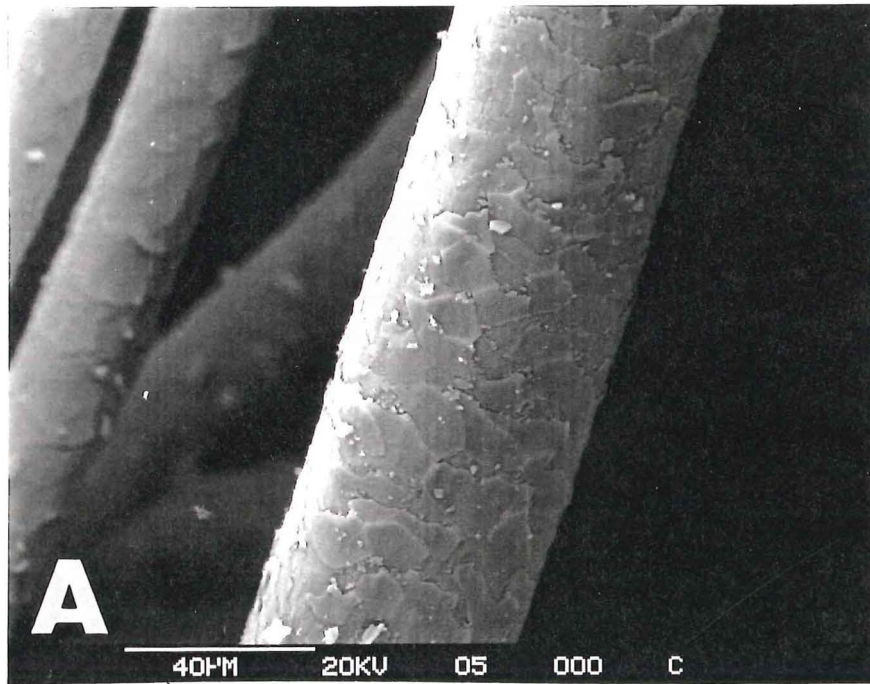




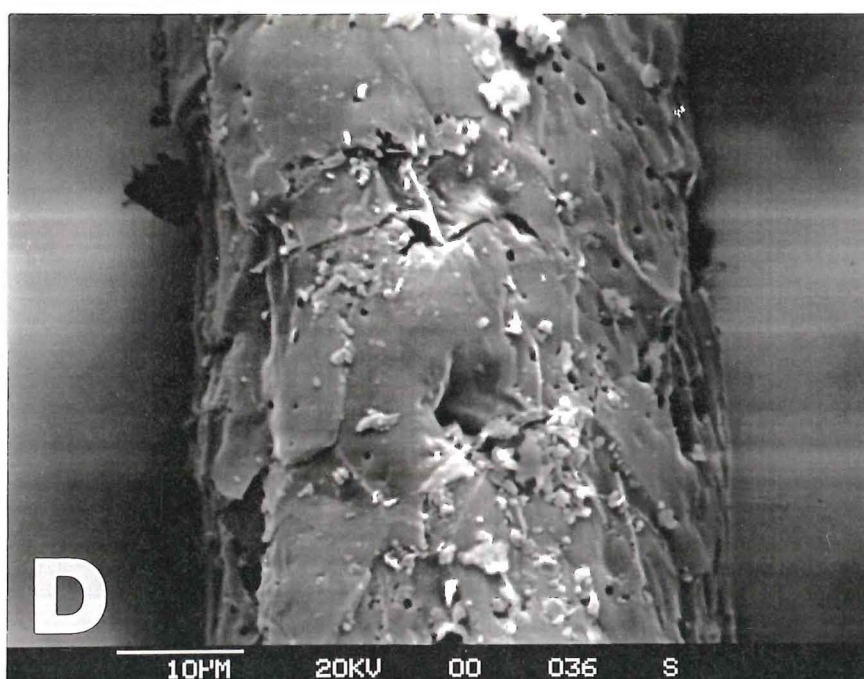
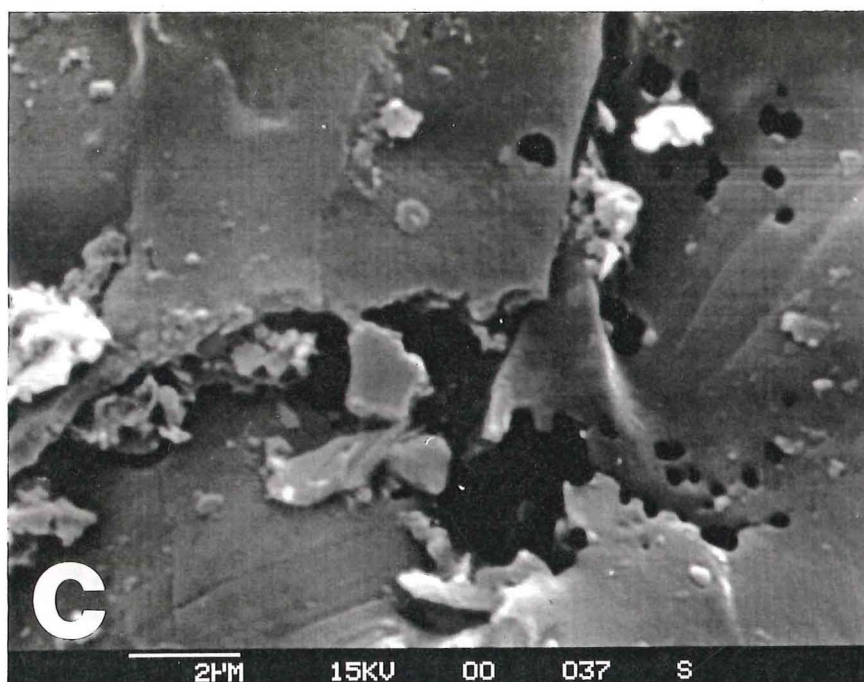


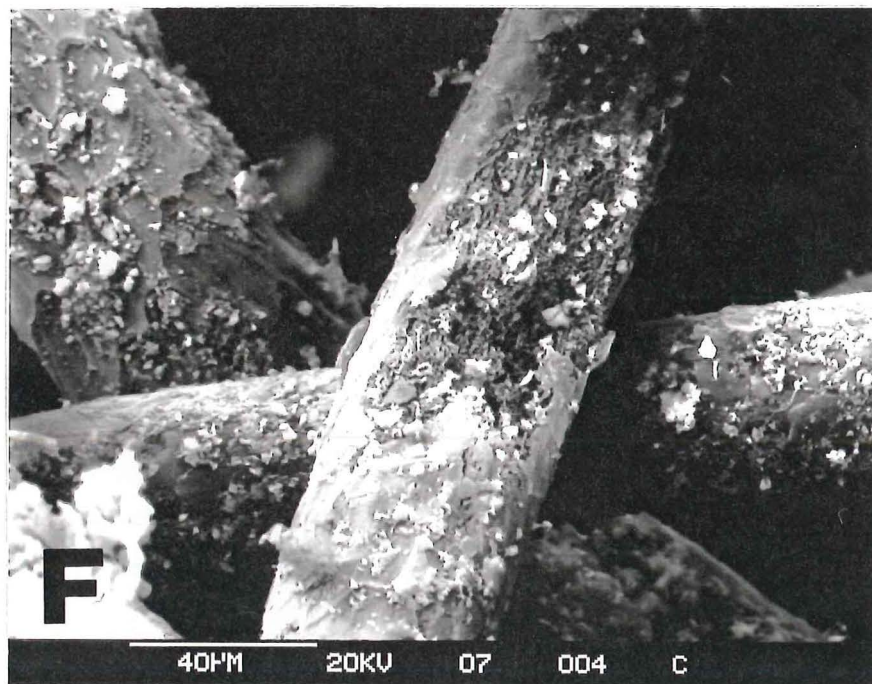
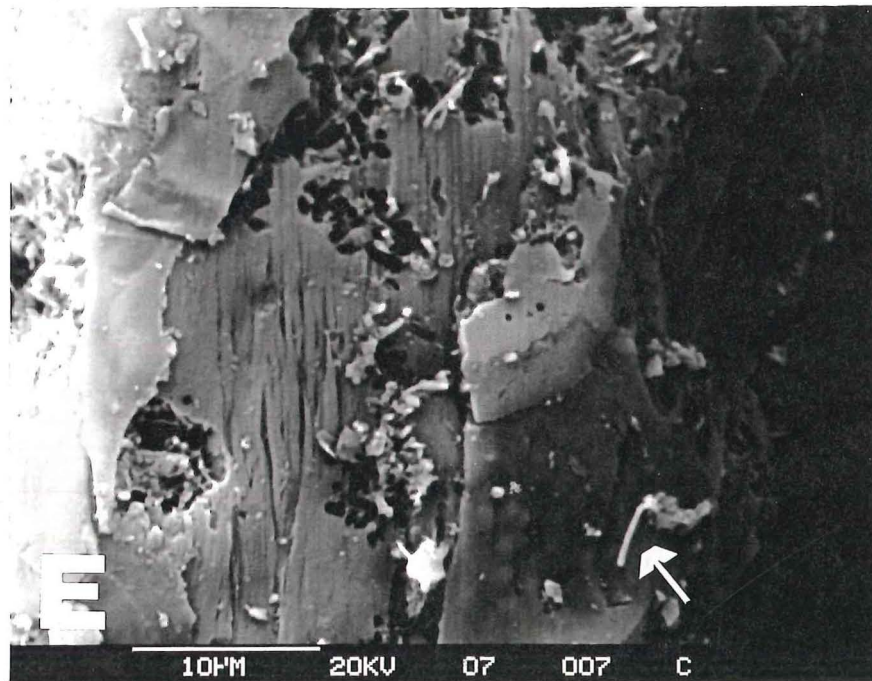
**Figure 14. Light Microscopy of Wool Fibres Removed from the Lyttleton Wool Oil Boom, at Monthly Intervals.**

Wool fibres stained with PAB showed various amounts of microbial growth and damage. At 0 months no microbial growth or damage was seen upon the wool fibres (A). At 1 month (B), and 2 months (C) microbial growth was seen as bacterial colonies upon the wool fibre. At 4 months, small pits were seen upon the wool fibres (D), while at 6 months, the pits had increased in size (E), and at 8 months most of the wool fibre was deeply pitted (F) (bar = 10  $\mu$ m).

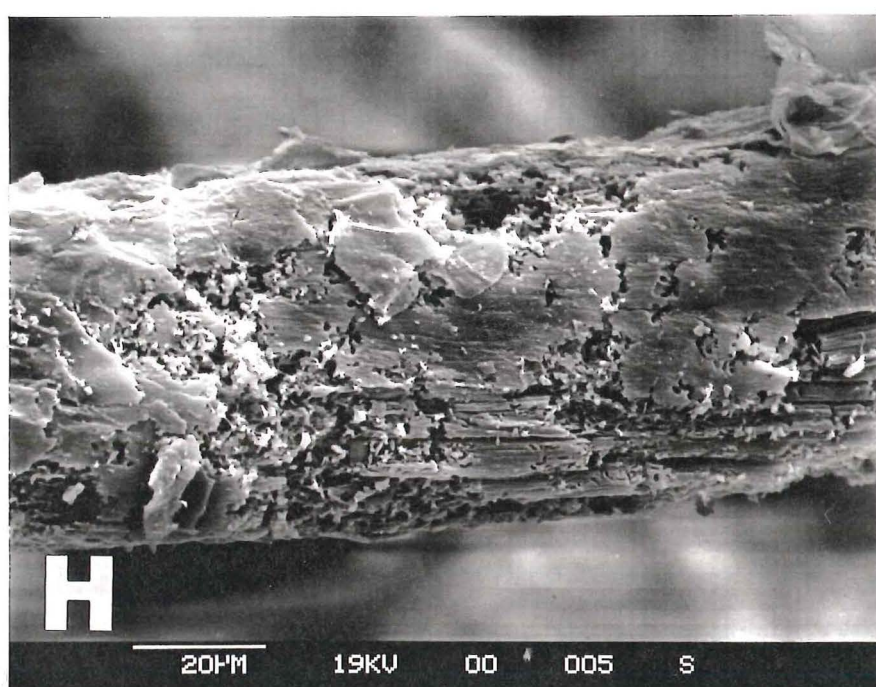
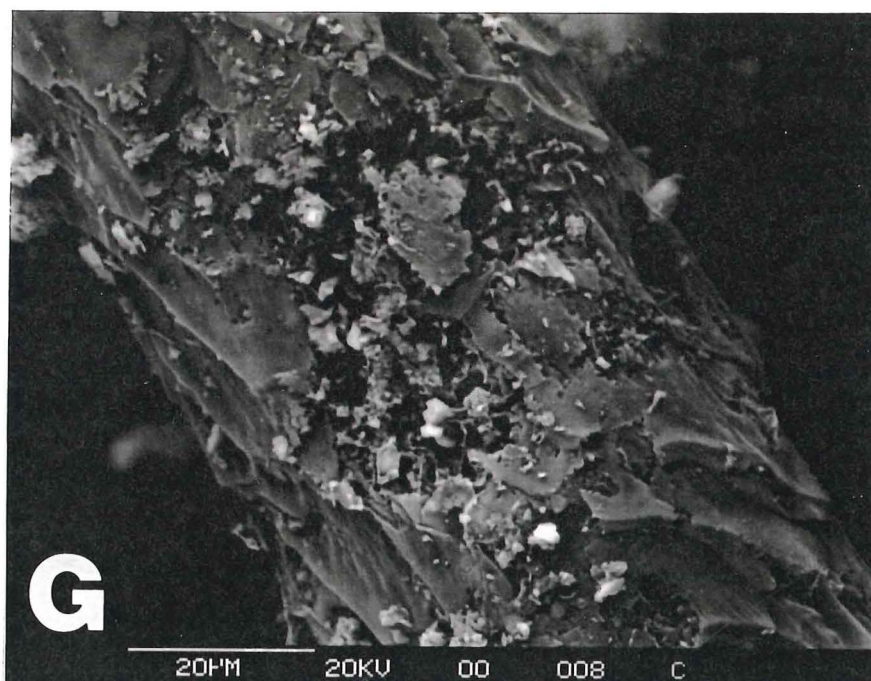


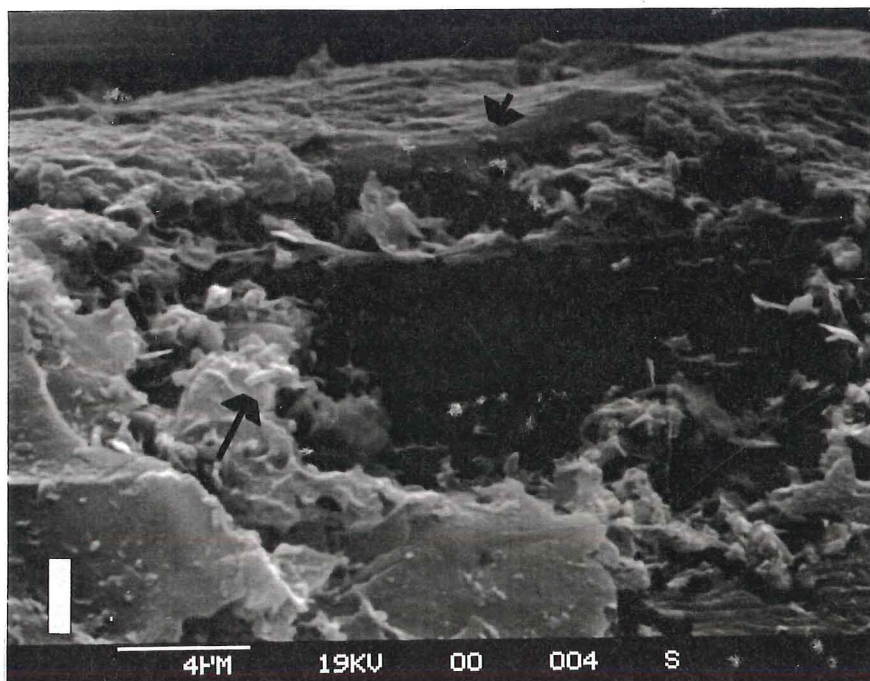












**Figure 15. Scanning Electron Microscopy of Wool Fibres Removed from the Lyttleton Wool Oil Boom at Monthly Intervals.**

SEM of the wool fibres removed from the wool oil boom, showed that wool fibre degradation was occurring. Before exposure to the marine environment, the fibres were undamaged (A). At 1 month, the fibres were mostly intact (B), although small holes had developed within some areas of the cuticle, and cuticle lifting was also noticeable (C). At 3 months, pitting had become more extensive (D), and in places the holes had penetrated through the cuticle, exposing the cortical cells (E). At 6 months, much of the cuticle on the wool fibre had been removed, exposing the cortical cells for degradation. In areas these cortical cells were beginning to be removed (G). At 8 months (H), the holes within the cortical cells had formed large pits, that appeared to extend deep into the fibre (I).

Rod Shaped Bacteria were regularly seen upon the wool fibres, especially in areas of damage (arrow).

### **3.3.4. Bacterial Analysis.**

#### **3.3.4.1. Assay of Proteolytic Activity.**

Proteolytic activity was indicated by the presence of a halo surrounding a bacterial colony, after the gelatin in the media has been precipitated by ammonium sulphate (Figure 16). The clear halo represents an area of degraded gelatin which has been hydrolysed by the extracellular proteolytic enzymes produced by the bacterial colony.

Of the 100 bacterial colonies randomly chosen from those isolated at monthly sampling intervals, proteolytic activity was found to be common. Between 76% and 83% of the bacterial colonies screened, were found to be proteolytic. The number of proteolytic bacteria isolated from the monthly wool knop samples are shown in Figure 17. The bacteria showing proteolytic activity appeared to be diverse with a number showing different colonial morphologies, and pigment production.

#### **3.3.4.2. Assay of Keratinolytic Activity.**

The proteolytic bacteria were screened for their ability to degrade KS, using the plate assay developed for screening marine bacteria. Initially 23% of the proteolytic bacteria screened were also capable of degrading KS. By 8 months the proportion of KS degrading bacteria had increased to 42% (Figure 17).

### **3.3.5. Actinomycete Analysis.**

No actinomycetes were isolated from wool knop samples during the sampling period.

### **3.3.6. Fungal Analysis.**

Fungal isolates were sporadically isolated from wool knop samples during the monthly sampling period (Table 2).

Proteolytic activity was common among fungal isolates. Fungal proteolytic activity upon MFGA plates were indicated in the same way as proteolytic activity shown by heterotrophic bacteria. No proteolytic fungal isolates showed the ability to degrade KS.

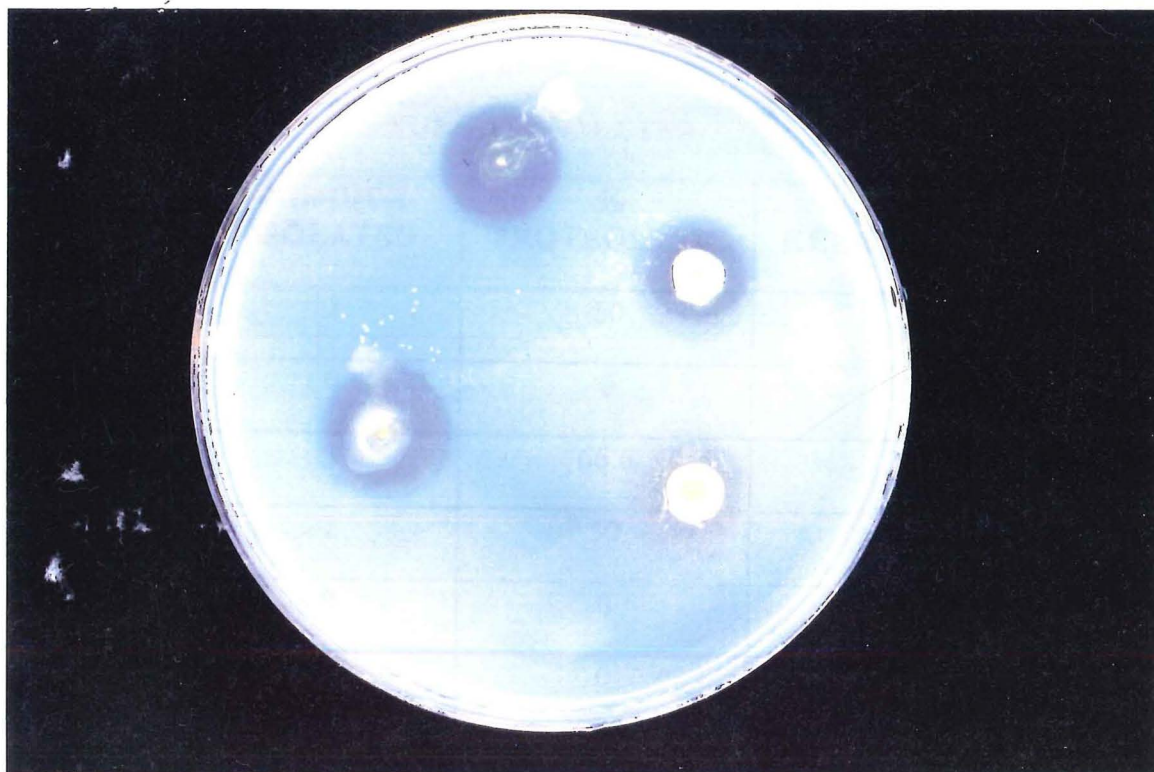
### **3.3.7. Tentative Identification of KS Degrading Bacteria.**

#### **3.3.7.1. Taxonomic Tests and API 20 NE Profiles.**

The results of the taxonomic tests which were performed upon the KS degrading bacteria are shown in Table 3.

These results showed that two different strains of bacteria capable of degrading KS, had been isolated. These were named marine bacteria A (MBA), and marine bacteria B (MBB). MBA was a gram negative, regular shaped rod, while MBB was a



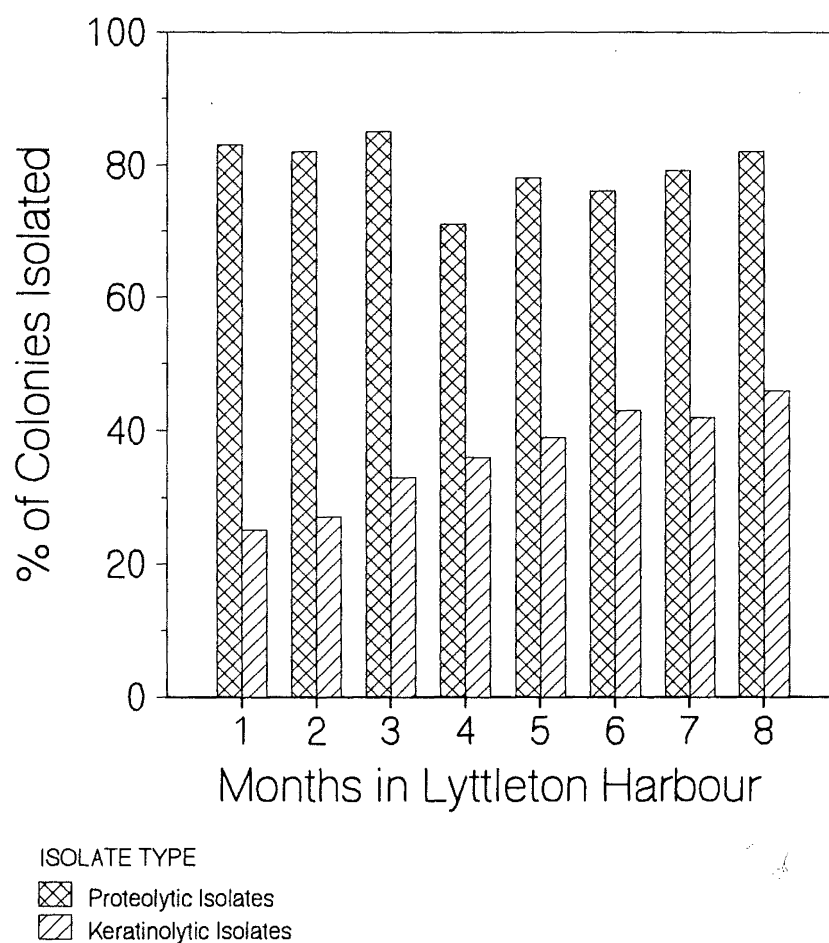


**Figure 16. Proteolytic Plate Assay.**

Proteolytic activity by marine isolates were indicated by a clear halo forming in the media around a bacterial colony after the gelatin had been precipitated from the media.

FUNGAL ISOLATES			
MONTH	NUMBER ISOLATED	% PROTEOLYTIC	% KERATINOLYTIC
1	2	50	0
2	0	0	0
3	3	66.6	0
4	2	50	0
5	0	0	0
6	0	0	0
7	3	66.6	0
8	2	50	0

**Table 2. Fungi Isolated from the Wool Oil Boom in Lyttleton Harbour, at Monthly Intervals.**



**Figure 17. The Percentage of Proteolytic and Keratinolytic Bacteria Isolated from the Wool Oil Boom at Monthly Intervals.**

The percentage of proteolytic bacteria remained relatively constant (between 76% and 83%), during monthly samplings. The percentage of keratinolytic bacteria however increased (from 23% to 42%), during the monthly samplings.



gram negative spiralled rod with between 1 and 3 spirals per bacterium. The frequency with which MBA was isolated appeared to increase over the monthly sampling periods, while the numbers of MBB decreased (Figure 18).

The adapted API 20 NE tests provided information on the metabolic activities of the isolated bacteria (Figure 20). Each species possessed different metabolic strains. These strains were named MBA1, MBA2, MBB1, MBB2, and MBB3. All MBA strains were able to metabolise gelatin, caprate, and produce oxidase, but variation between strains existed in their ability to metabolise arginine, urea, esculin, and maltose. For MBB, all strains were able to metabolise gelatin, p-nitro-phenyl- $\beta$ D-galactosidase, mannitol, and caprate, but variation between strains existed for esculin, arabinose, mannose, and maltose. The frequency with which each strain was isolated had no correlation with time.

### 3.3.7.2. TEM.

Negative staining of bacteria MBA, and MBB, revealed that both isolates possessed a single polar flagellum. Problems were occasionally encountered with cells of MBB, as they often appeared wrinkled and invaginated. This may be due to the use of distilled water during negative staining, therefore causing a disruption in osmotic pressure within the cells.

### 3.3.7.3. Fatty Acid Analysis and Identification.

The fatty acid analysis profiles of MBA and MBB, are shown in Table 4. Comparison of the fatty acid profiles of bacterial species already analysed by the Microbial ID Inc. database indicated that MBA was a species not found in the database. The closest related species found in the database was *Alteromonas haloplanktis*. MBB was identified as a good match to *Acinetobacter iwoffii*.

Isolate identification is based upon a similarity index. This is a numerical value which represents how closely the fatty acid analysis of an unknown isolate compares with the mean fatty acid composition of known strains. Strains of a similarity index of 0.500 or higher, and with a separation of 0.100 between first and second choice are good matches. A similarity index between 0.300 and 0.500 may be a good match but would indicate an atypical strain. Values lower than 0.300 suggests the species is not in the database, but those listed provide the most closely related species (Microbial ID Inc., personal communication).

### 3.3.8. Confirmation of Wool Biodegradation.

#### 3.3.8.1. Keratin Azure Biodegradation.

All bacterial isolates capable of degrading KS, were able to liberate blue stain from Keratin Azure indicating degradation of the Keratin Azure fibres (Figure 21).

ISOLATES	MBA	MBB
COLONY COLOUR	Yellow	Cream
GRAM STAIN	Gram negative, regular rods	Gram negative, curved or spiralled rods
KERATINOLYTIC	+	+
PROTEOLYTIC	+	+
CATALASE	+	+
MOTILITY	single polar flagellum	single polar flagellum

Modified API 20 NE Strips	MBA1	MBA2	MBB1	MBB2	MBB3
Nitrate	-	-	-	-	-
Tryptophan	-	-	-	-	-
Glucose	-	-	-	-	-
Arginine	-	+	-	-	-
Urea	-	+	-	-	-
Esculin	+	-	-	-	+
Gelatin	+	+	+	+	+
p-nitro-phenyl- $\beta$ D-galactosidase	-	-	+	+	+
Glucose	-	-	-	-	-
Arabinose	-	-	-	+	-
Mannose	-	-	-	+	+
Mannitol	-	-	+	+	+
N-acetyl-glucosamine	-	-	-	-	-
Maltose	+	-	-	-	+

Gluconate	-	-	-	-	-
Caprate	+	+	+	+	+
Adipate	-	-	-	-	-
Malate	-	-	-	-	-
Citrate	-	-	-	-	-
Phenyl-acetate	-	-	-	-	-
Oxidase	+	+	-	-	-

<b><u>FATTY ACID ID</u></b>	<i>Alteromonas (?)</i>	<i>Acinetobacter iwoffii</i>
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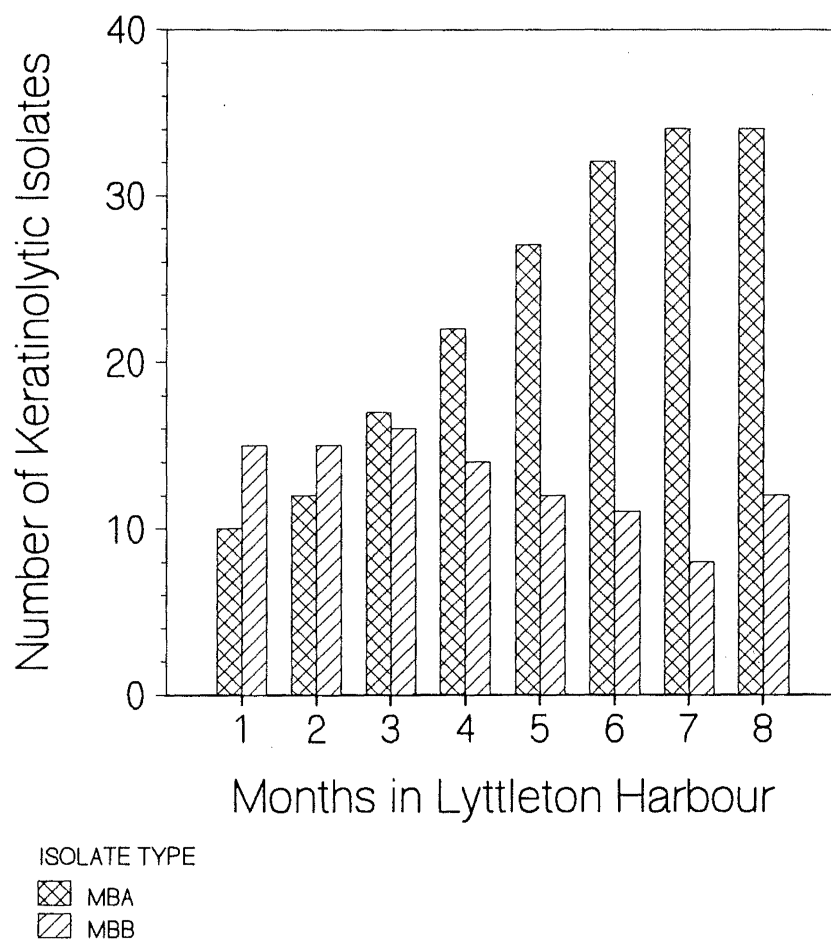
<b><u>TENTATIVE ID</u></b>	<i>Alteromonas</i>	<i>Oceanospirillum</i>
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+ positive, - negative

**Table 3. Taxonomic Analysis of KS Degrading Bacteria, Isolated from Lyttleton Harbour.**

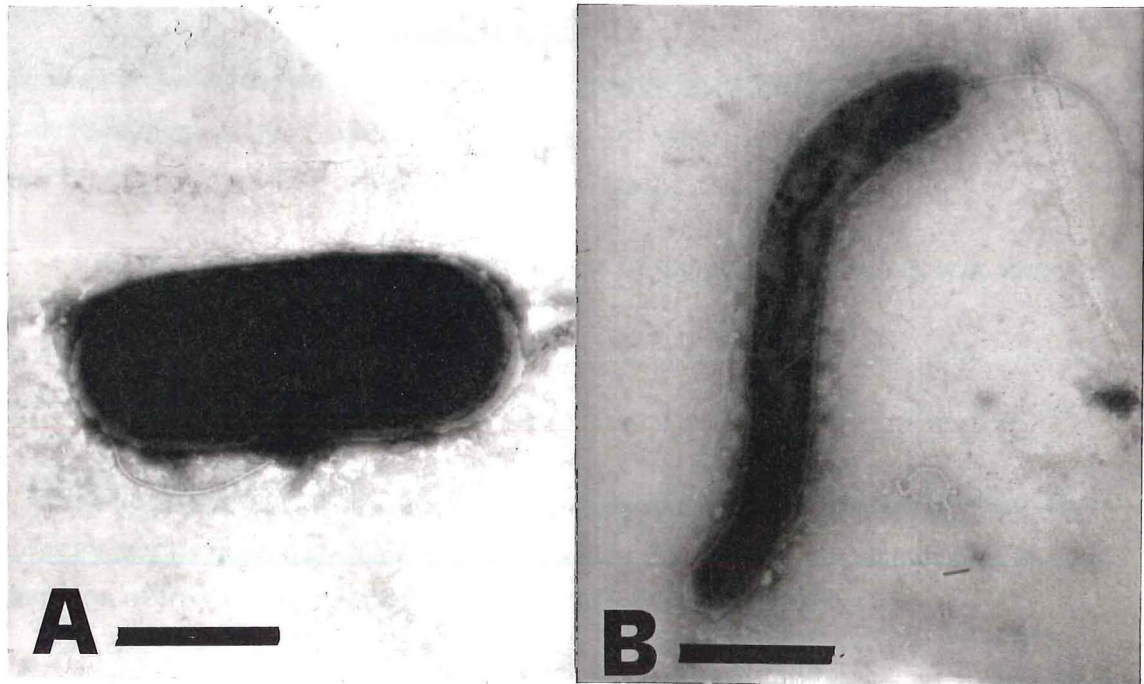
ISOLATE	IDENTIFICATION	SIMILARITY INDEX	SIGNIFICANCE
<b>MBA</b>	<i>Alteromonas haloplanktis</i>	0.013	Related species
<b>MBB</b>	<i>Acinetobacter iwoffii</i>	0.708	Good match. Probable identification.

**Table 4. The Fatty Acid Analysis of Isolates MBA and MBB.**



**Figure 18. The Number of MBA and MBB Bacteria, Isolated from the Wool Oil Boom at Monthly Intervals.**

KS degrading bacteria MBA, and MBB varied in number at different sampling periods. MBA increased in numbers as time increased, while MBB decreased in numbers as time increased.



**Figure 19. Transmission Electron Microscopy of Isolates MBA and MBB.**

Isolates MBA, and MBB varied greatly in their cellular morphology. MBA was a regular rod with polar flagellum, while MBB was a spiralled rod, with single polar flagellum (bar = 0.5  $\mu\text{m}$ ).

Stain release was first noticeable at approximately 1 month for all isolates, and continued over subsequent months. Cleavage of the Keratin Azure fibres began after 4 months incubation. Neither stain release, or fibre cleavage was seen in the sterile controls.

After 5 months incubation pure cultures of the original inoculum was able to be reisolated from the cultures.

#### **3.3.8.2. Wool Biodegradation.**

Bacteria capable of degrading KS in plate assays were capable of degrading wool. After 1 month growth in wool knops, attachment of the bacteria was observable under light microscopy. After 5 months small pits were seen in the wool fibres. No microbial growth or fibre damage was seen on sterile controls.

After 5 months incubation pure cultures of the original inoculum was able to be reisolated from the cultures.

### **3.4. EFFECT OF OIL ON THE BIODEGRADATION OF WOOL.**

The adsorption of oil onto wool decreased the rate of degradation of the wool fibres by marine microbes. This was seen by light microscopy, and showed in the liberation of free amino groups from the wool (Figure 22).

#### **3.4.1. Free Amino Group Release.**

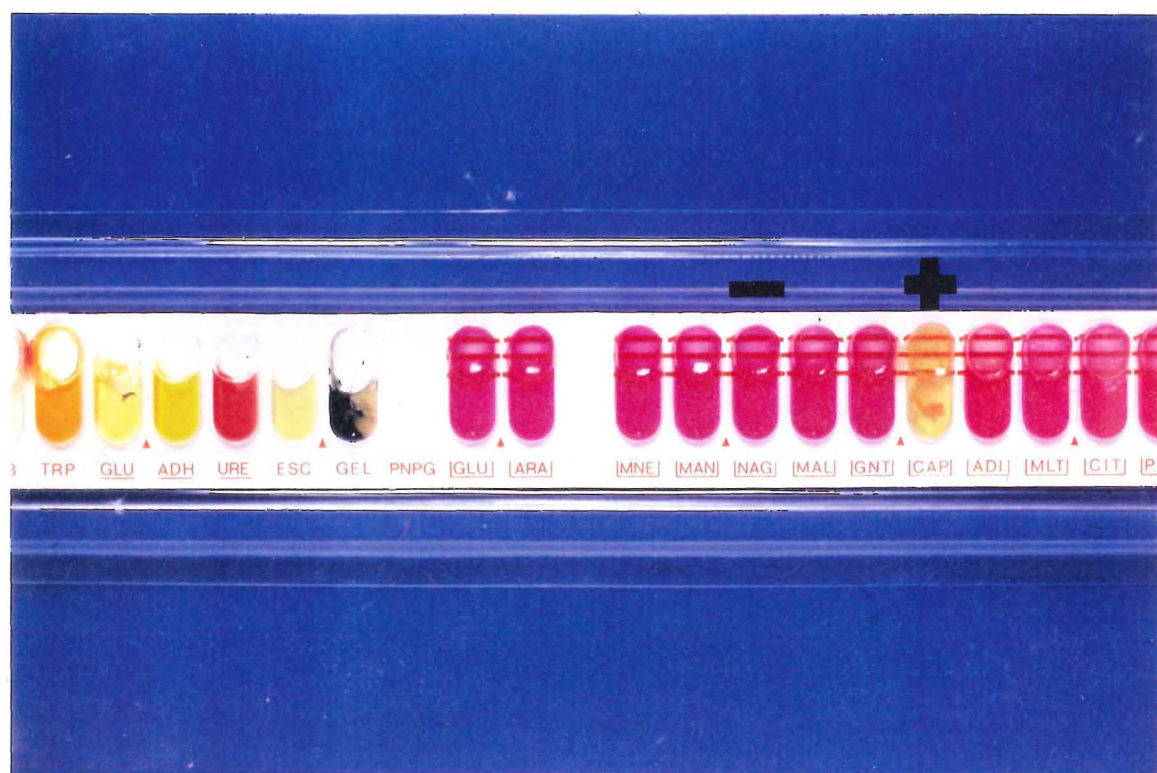
For the cultures of mixed marine microbes, the rate of free amino group release decreased when the wool fibres were coated with oil. Unlike the keratinolytic controls, the level of free amino group release in the oil and wool culture never reached that of the level of the wool culture, after 32 weeks.

Similar results were seen for the keratinolytic bacterial controls of *Lysobacter* and *S. fradiae*, with the rate of free amino group release reduced by the addition of oil to the culture. Peak free amino group release was extended from 21 days to 60 days for *S. fradiae*, and from 28 days to 70 days for *Lysobacter*.

None of the uninoculated controls containing wool in sterile sea water, or wool in sterile LBM or SFBM, exhibited free amino group release.

#### **3.4.2. Microscopic Examination of the Wool Fibres.**

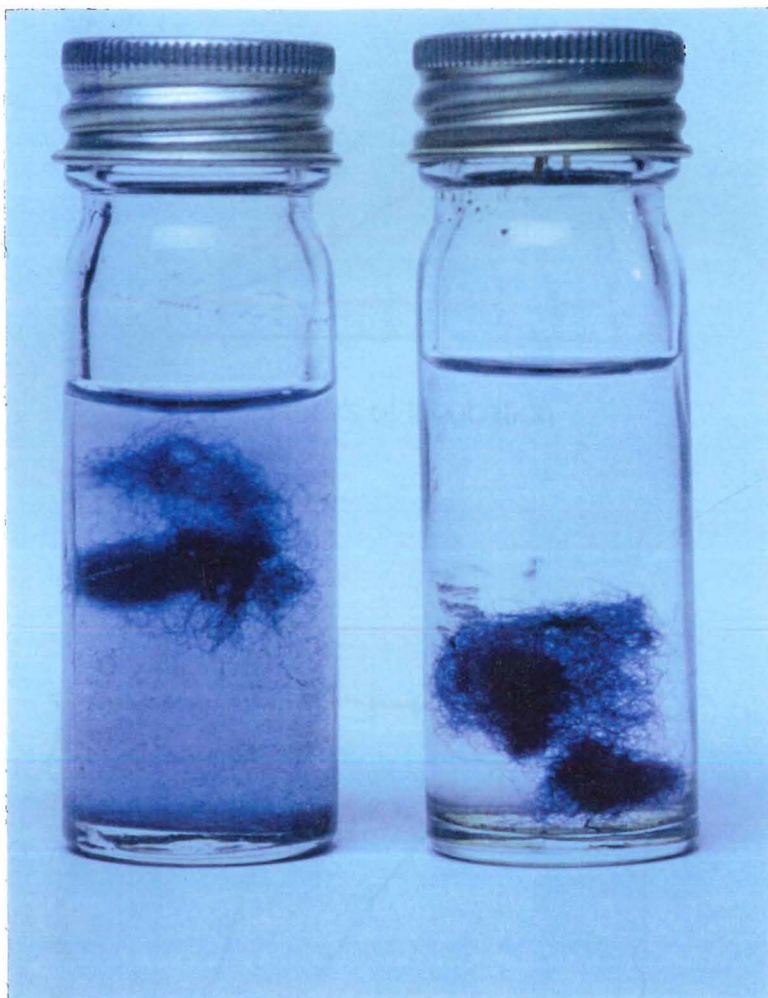
Light microscopy further supported the biochemical data, indicating that wool fibre degradation decreased when oil was adsorbed upon the wool fibres. At 32 weeks wool fibres from the mixed cultures of marine microbes, were absent in pitting the



**Figure 20. An API 20 NE Strip Adapted for use with Marine Bacteria.**

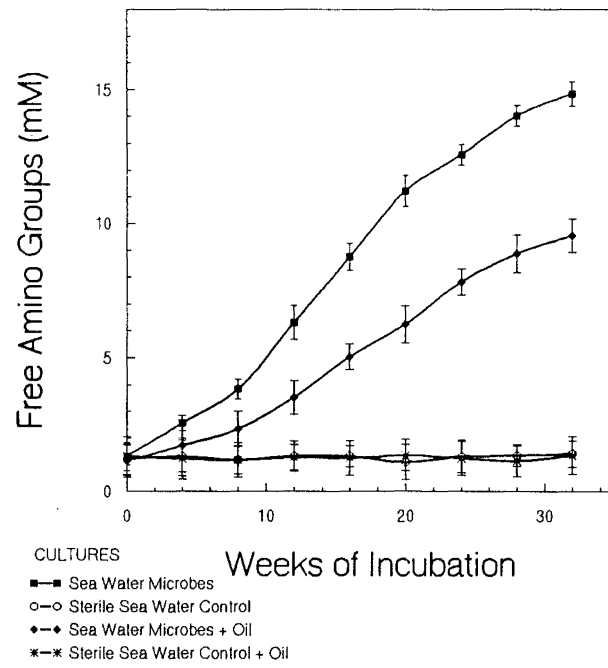
The use of phenol red in MPRBA allowed an easier interpretation of a positive result. Positive results were indicated by a change in colour of the cupule media from pink (-) to yellow (+), indicating that acid products were being produced, and therefore the assimilation of the substrate in the cupules.



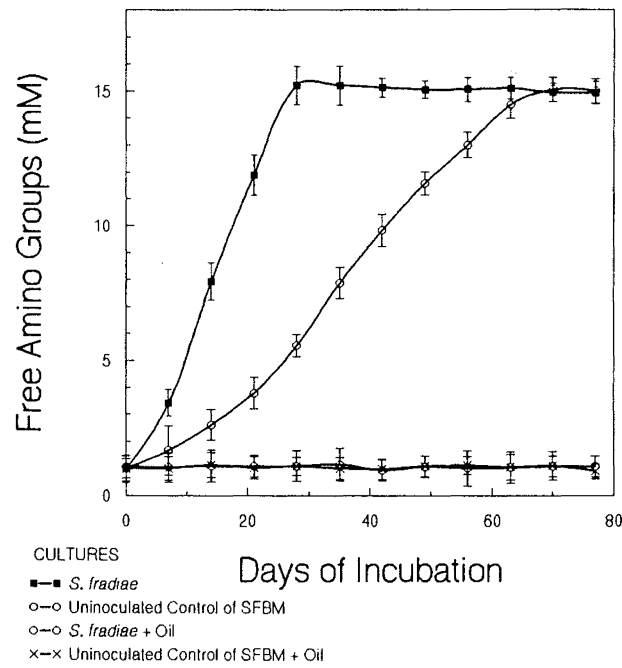


**Figure 21. Stain Liberation from Keratin Azure by the Marine Isolate MBA.**

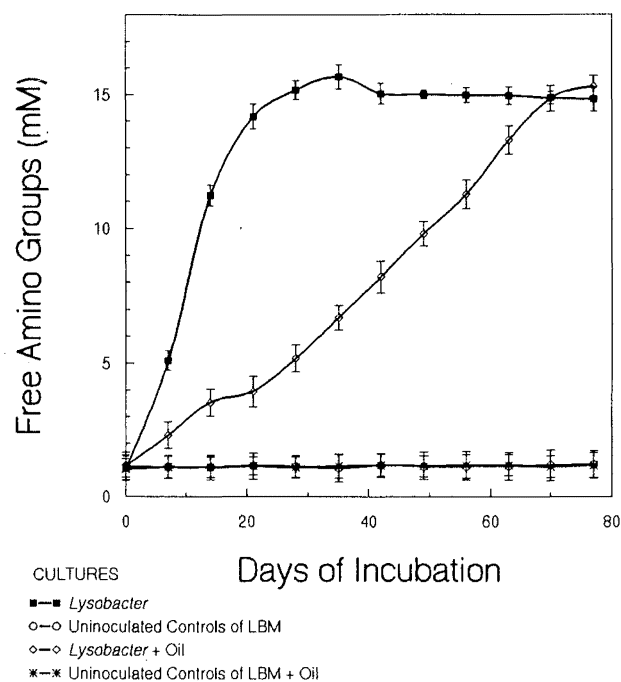
Stain liberation from Keratin Azure can be seen after 5 months incubation with MBA, by the blue colour of the surrounding liquid media (left), while no stain is release is seen in the uninoculated control (right).



A



B



C

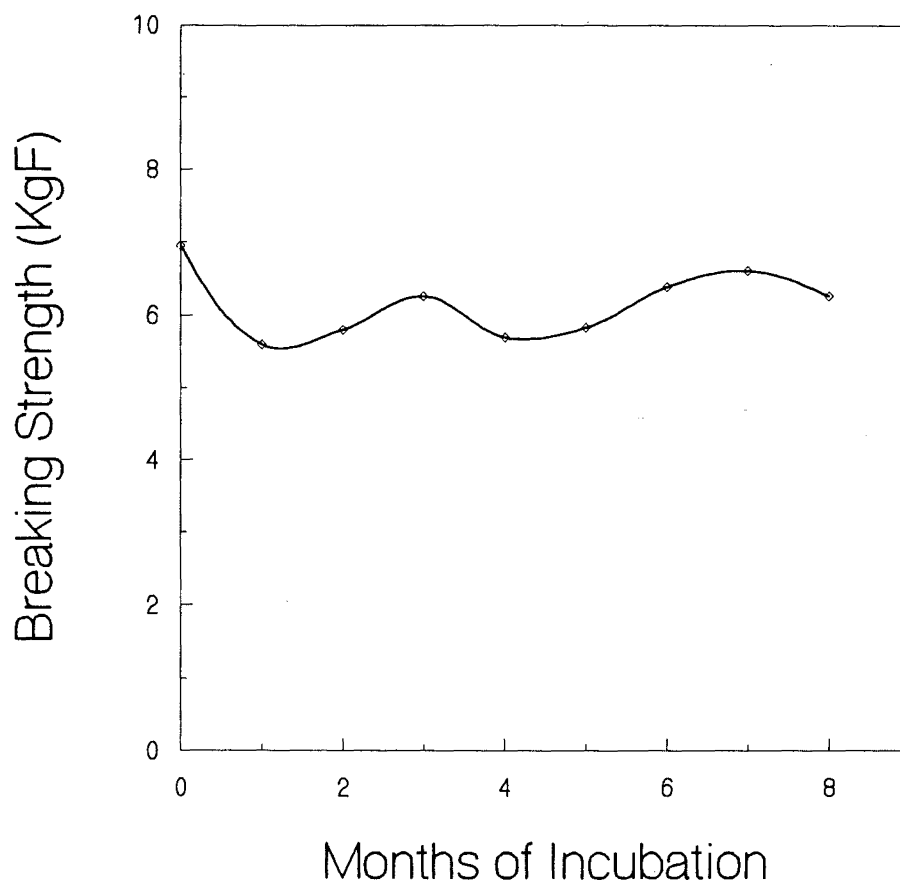
**Figure 22. Free Amino Group Release During the Degradation of Wool With Oil Adsorbed, by Cultures of Sea Water Microbes, and Keratinolytic Bacteria.**

The rate of free amino group release was greatly reduced when oil was absorbed onto wool. In cultures of the microbes in sea water, a large reduction in degradation occurred, and after 32 weeks the level of free amino group release from the wool with oil adsorbed had not reached the same level as that without oil (A). The keratinolytic bacteria of *S. fradiae* (B), and *Lysobacter* (C), also showed a reduction in the rate of free amino group release. In both cases however, free amino group release was achieved to a level similar to that seen in cultures without oil.

wool fibres from oil containing cultures, while at the same time it was prevalent upon wool fibres not containing oil. No wool fibre damage was seen on fibres removed from the control flasks. Similar results were seen for the cultures of *Lysobacter* and *S. fradiae*, with the release of cortical cells into the culture solution occurring later than that seen in cultures without oil.

### **3.5. EFFECT OF ABIOTIC FACTORS IN SEA WATER ON WOOL STRENGTH.**

When woollen fabric was incubated in sterile sea water no effect was found in reducing the breaking strength of woollen fabric (Figure 23). Little variation existed between monthly fabric samples.



**Figure 23. The Effect of Sterile Sea Water on Wool Strength.**

Little variation was seen in the strength of woollen fabric when exposed to sterile sea water. This indicates that abiotic factors in sea water are not responsible for the degradation of wool in sea water.

## 4. DISCUSSION.

### 4.1. ASSAYS FOR WOOL DEGRADATION.

#### 4.1.1. Biodegradation of Wool *In vitro*.

Enrichment cultures of marine bacteria from sea water, provided with wool (in the form of wool knops or Keratin Azure) as a carbon and nitrogen source, were established. Wool degradation was monitored by the use of biochemical indicators, released during wool degradation under controlled conditions, in the laboratory. This was done to establish whether biodegradation of wool occurred in sea water.

##### 4.1.1.1. Keratin Azure Biodegradation.

Keratin Azure, a woollen substrate comprised of lambs wool dyed with the biological stain Azure, proved useful for analysing the ability of microbes to degrade wool in liquid culture. This was achieved by monitoring stain release, and observing wool fibre damage was able to be monitored.

Stain release was able to be quantitatively measured for the mixed cultures of marine microbes, and for the keratinolytic bacteria of *S. fradiae* and *Lysobacter*. The degradation and stain release of Keratin Azure, by the microbes from sea water, was considerably slower than that seen in the cultures of the positive keratinolytic control bacteria. Degradation of the wool fibres, observed under light microscopy, was similar to that seen in the degradation of wool oil booms in Lyttleton Harbour.

The process of Keratin Azure degradation was similar to that seen in the wool oil booms, with pitting in the wool fibres occurring after 12 weeks, and large pits appearing in the fibres at 32 weeks. Degradation of Keratin Azure fibres by *S. fradiae* and *Lysobacter* was of a very different nature. Degradation of these fibres took the form of that seen in other studies of wool degradation by *S. fradiae* (Carter *et al.*, 1987; Brady *et al.*, 1990), with cortical cell splinters being released from the wool fibres. After maximum stain release the Keratin Azure fibres had been degraded down to a stage where only cortical cells remained in the culture solution. *Lysobacter*, like *S. fradiae*, attacks the non-keratinous substances in the wool fibre first, and therefore releases the keratinous cortical cells from the wool fibre. No previous studies have been conducted on the mechanism that this bacterium possesses to degrade wool, except to note that it is enzymatic (Napier, 1966).

The same level of stain release that was achieved in the keratinolytic bacterial cultures were not seen to occur in the sea water cultures. This is due to the fact that the Keratin Azure fibres in the mixed cultures of marine microbes were not degraded to the same extent as those in the pure cultures of *S. fradiae* and *Lysobacter*. This may be due to the different mechanisms of wool degradation, as the positive keratinolytic bacterial

controls were more extensive in their degradation of the non-keratinous cuticle upon the wool fibre, while microbes in the sea water cultures were more localised in their action. Differences in levels of stain liberation may also have been caused by differences in cell numbers within the cultures, and also the use of different culturing conditions.

#### 4.1.1.2. Wool Biodegradation.

Wool degradation was monitored in three ways, by free amino group release, release of soluble sulfhydryl concentrations, and microscopic examination of wool fibres.

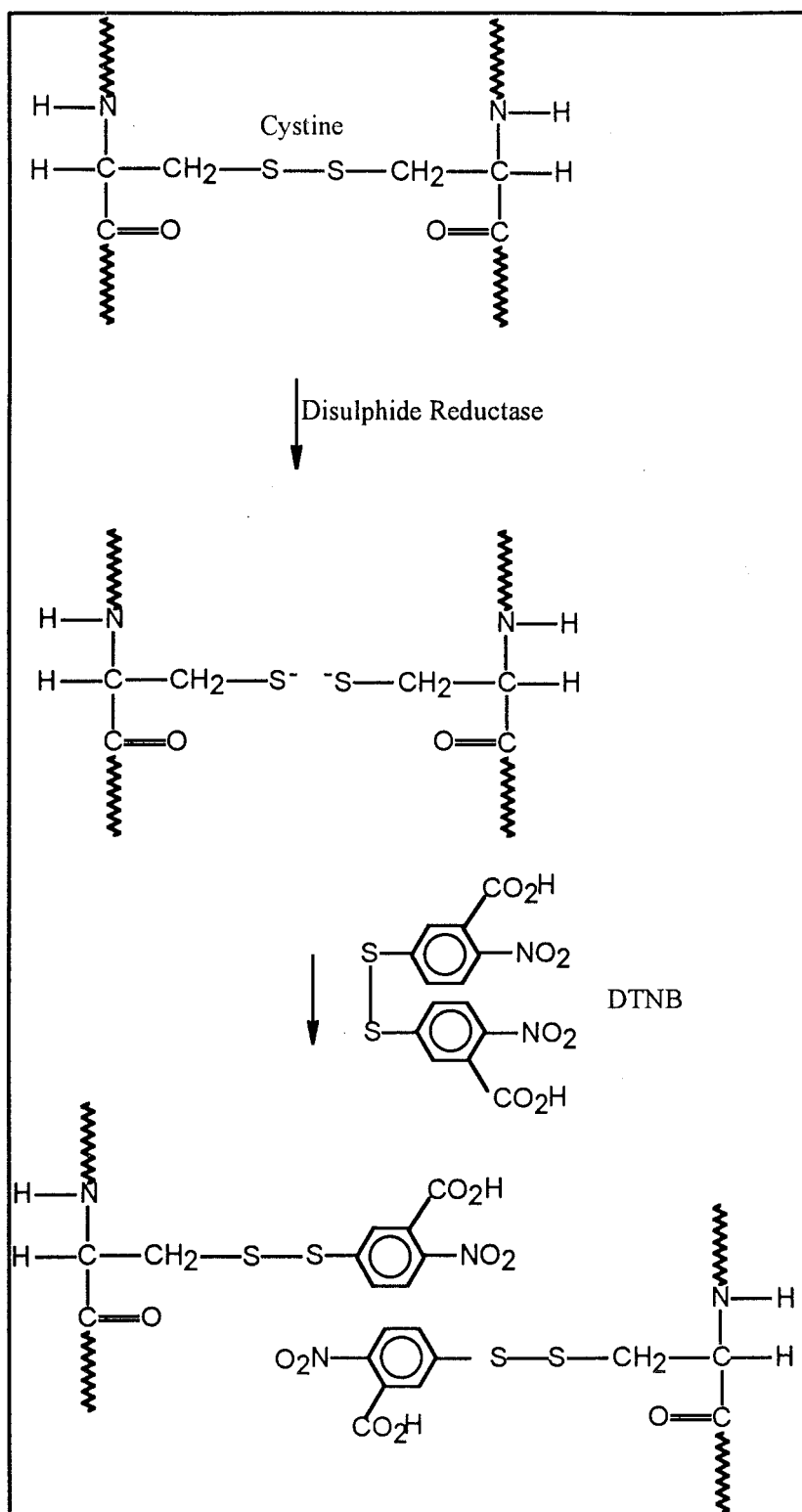
Free amino groups occur due to the cleavage of the peptide bonds when a protein is degraded. These amino groups react with ninhydrin producing a coloured product which can be colorimetrically monitored at 440 or 570 nm using a spectrophotometer (Rosen, 1957).

The analysis of free amino groups in the culture solutions of the keratinolytic bacteria *S. fradiae* and *Lysobacter*, and the mixed culture of marine microbes, showed that free amino groups were being released as the wool fibre proteins were degraded. Free amino groups have been used previously to monitor degradation of autoclaved feathers by enzymes of a feather degrading *Bacillus lichenformis* strain (Xiang *et al.*, 1992). The keratinolytic control bacteria, released free amino acid groups much more rapidly than the mixed cultures of marine microbes. As was seen in the liberation of stain from Keratin Azure, free amino group release by the mixed culture of marine microbes was slightly less, after 32 weeks incubation, than that which occurred in the keratinolytic control bacteria.

The analysis of soluble sulfhydryl concentrations in the liquid cultures produced some interesting results. It is generally agreed that soluble sulfhydryls should be produced during keratin degradation due to the breakage of the resistant disulphide bonds that occur in keratin (Mathison, 1964). This is postulated to occur via the reduction of the cystine molecule, by a microbial disulphide reductase, at the disulphide bond (Figure 24).

The analysis of soluble sulfhydryls concentrations in the monthly culture samples revealed that no soluble sulfhydryls were detectable in the mixed culture of marine microbes. This indicates that the degradation of wool by marine bacteria does not involve the degradation of wool keratin by the breakage of the disulphide bonds, such as that which occurs by the use of a disulphide reductase. A similar result was found in the study of keratin degradation by the enzymes liberated by the feather degrading *Bacillus lichenformis*. The microbe was unable to produce soluble sulfhydryls during feather





**Figure 24. The Disruption of a Disulphide Bond, and the Reaction of the Resulting Soluble Sulphydryls with Ellman's Reagent.**

A disulphide bond in a cystine molecule within keratin, is broken by a disulphide reductase from a keratinolytic microorganism. The resulting soluble sulphydryls are then able to be assayed for using colorimetry, as DTNB oxidises the reduced sulphydryl groups, resulting in the production of a yellow coloured compound.

degradation (Xiang *et al.*, 1992). In this study however, feathers which had been autoclaved and ground were used, which may have meant that the disulphide bonds in the feather keratin had already been disrupted, as it has been shown that this kind of treatment weakens keratin for microbial degradation (Noval and Nickerson, 1959). No soluble sulfhydryl production by sea water microorganisms indicates that either keratin degradation is not occurring, or the mechanism for degrading keratin does not involve the release of soluble sulfhydryls. Alternatively, soluble sulfhydryl groups which were released by keratin degradation, may have been rapidly metabolised by the marine microbes within the mixed cultures of marine microbes. This is unlikely as sulphur is not limiting, due to the presence in high concentrations of sulphate.

Similar concentrations of soluble sulfhydryls were released in cultures of both *Lysobacter* and *S. fradiae*. This has been recorded previously for cultures of *S. fradiae* (Noval and Nickerson, 1959). In *Lysobacter*, the production of soluble sulfhydryls has not been studied.

Whether the production of soluble sulfhydryls is an indication of keratinolytic activity is, as stated previously in the introduction, a contentious point, due to the lack of reproducibility of such results with cell free filtrates (Brady *et al.*, 1990). It therefore appears that the release of sulfhydryls by *S. fradiae*, may not be an indication of disulphide bond disruption, but rather due to some other process, such as the metabolism of components within the media culture. Such results indicated that *S. fradiae* may not be capable of degrading the keratin component of wool, as suggested by microscopy, but rather the non-keratinous proteins within the wool fibre. If *S. fradiae* is unable to degrade keratin, it throws doubt upon the labelling of this organism as keratinolytic, and a more correct label when discussing this bacterium would be wool degrading.

Examination of wool fibre degradation by light microscopy, shows that wool fibre damage is proportional to free amino group release, in the liquid cultures of mixed marine microbes, and with free amino groups and soluble sulfhydryl release in the keratinolytic bacterial cultures. Degradation of the wool fibres followed the same pattern as was seen in the degradation of Keratin Azure, with the keratinolytic control bacteria degrading the fibre via the release of cortical cells, and the mixed cultures of marine microbes, by fibre pitting.

#### **4.1.2. Development of a Keratinolytic Plate Assay.**

Through the study of wool biodegradation *in vitro* it was observed that wool was capable of being degraded by the microbes present in sea water. The next step was to isolate the microbes responsible for degrading wool from sea water. Prior to the isolation of marine bacteria, an assay was required to rapidly screen isolates for their wool degrading ability. The use of KS in a plate assay to screen microbes for their wool

degrading ability proved successful. KS degradation was shown by a clear halo forming in the KS overlay in areas surrounding bacterial colonies. This indicated that the bacteria possessed enzymes with the ability to hydrolyse the KS in these haloes. KS has been used previously for screening the keratinolytic activity of fungal dermatophytes (Wawrzekiewicz *et al.*, 1991), and also for characterising the keratinases of the fungus *Trichophyton gallinae* (Wawrzekiewicz *et al.*, 1987). KS has not been used previously to screen bacteria for keratinolytic activity.

The structure of KS used in this assay was not studied, and the effect of solubilising keratin in DMSO is unknown. In a previous study (Wawrzekiewicz *et al.*, 1987) it was found that the chemical composition of KS, prepared using DMSO, was similar to that of native keratin, however it was found that the KS had a higher level of  $\alpha$ -amino nitrogen (Table 5). This suggests that the substrate was partially degraded.

In experiments carried out by Wawrzekiewicz *et al.* (1987) it was found that although KS was degradable, native wool keratin was not necessarily degraded. This may be attributed to the partial degradation of keratin in KS by DMSO, as discussed above. Alternatively the feather keratin used to produce KS has slight chemical and physical differences to wool keratin. It was therefore essential that additional experiments be carried out, such as culturing the bacterium in liquid cultures with wool substrates, so that the KS degrading bacterium can be labelled as wool degrading.

The use of KS for screening microbial isolates for wool degrading ability provides a rapid plate assay. KS also provides additional advantages, for example wool, feathers, horn and other keratin containing substrates, can not be used in plate assays, and require stringent sterilisation techniques, such as ethylene oxide in order not to denature the keratin protein within the substrate, that can occur with autoclaving (Noval and Nickerson, 1959). The extraction of KS from wool was also attempted during this study however, this was unsuccessful.

## **4.2. BIODEGRADATION OF WOOL *IN SITU*.**

The placement of a wool oil boom in Lyttleton Harbour allowed the enrichment of marine microbes capable of degrading wool. It also allowed the degradation of wool fibres contained in the wool oil boom to be observed through microscopy.

### **4.2.1. Wool Boom Biodegradation.**

It was apparent from monthly observations of the wool oil boom that had been exposed to the marine environment in Lyttleton Harbour, and subsequent microscopic observations, that the wool knops in the boom were being degraded. Although total

<b>CHEMICAL COMPOSITION</b>	<b>UNMODIFIED CHICKEN FEATHER KERATIN</b>	<b>KERATIN PREPARATION WITH DMSO</b>
<b>Dry Matter</b>	95.3	91.3
<b>Total Protein</b>	94.5	90.5
<b>Ether Extract</b>	0.4	0.3
<b>Fibre</b>	0.2	0.1
<b>Ash</b>	0.2	0.1
<b><math>\alpha</math>-Amino Nitrogen (in mg per 100 mg of dry matter)</b>	0.45	0.7

**Table 5. The Chemical Composition of Soluble Keratin, and Keratin.**

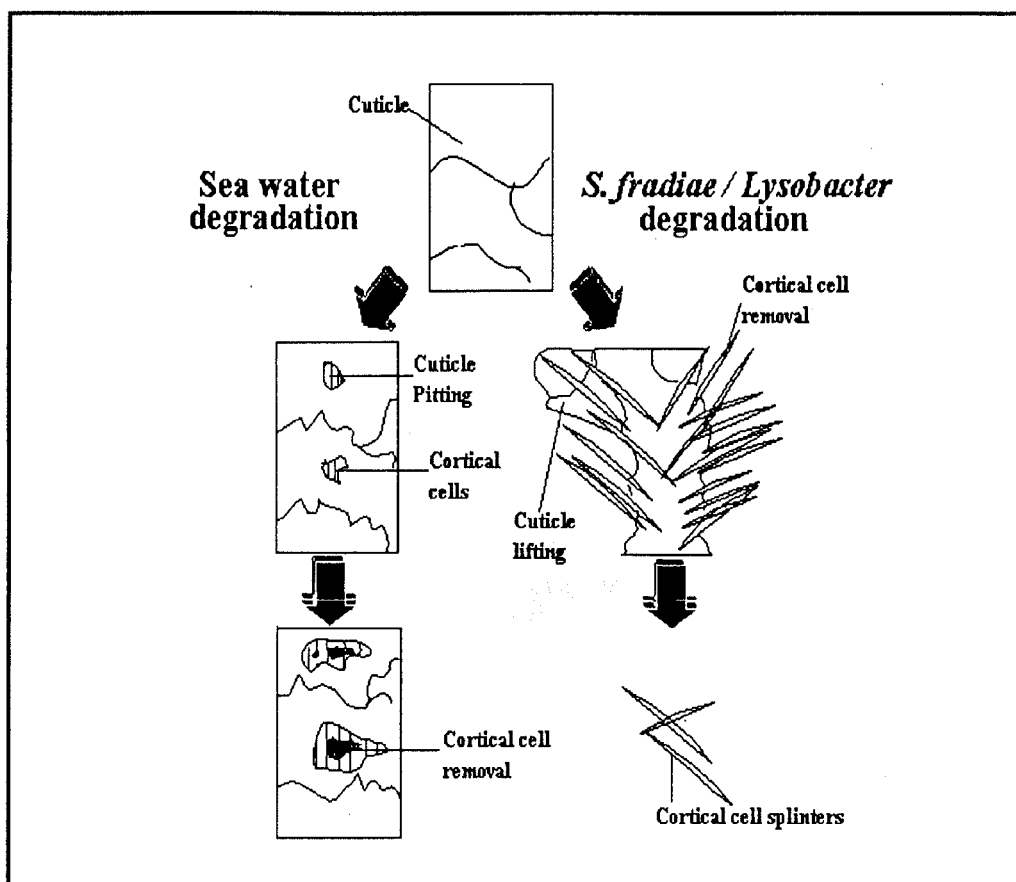
(From Wawrzekiewicz *et al.*, 1987).

wool loss was not achieved from the wool boom after 8 months, the wool fibres were considerably degraded.

Degradation of wool fibres in the marine environment appears to involve a process in which the first step involves the formation of a biofilm upon the wool fibres. After this the cuticle is degraded, and removed from the fibres, exposing the cortical cells for degradation.

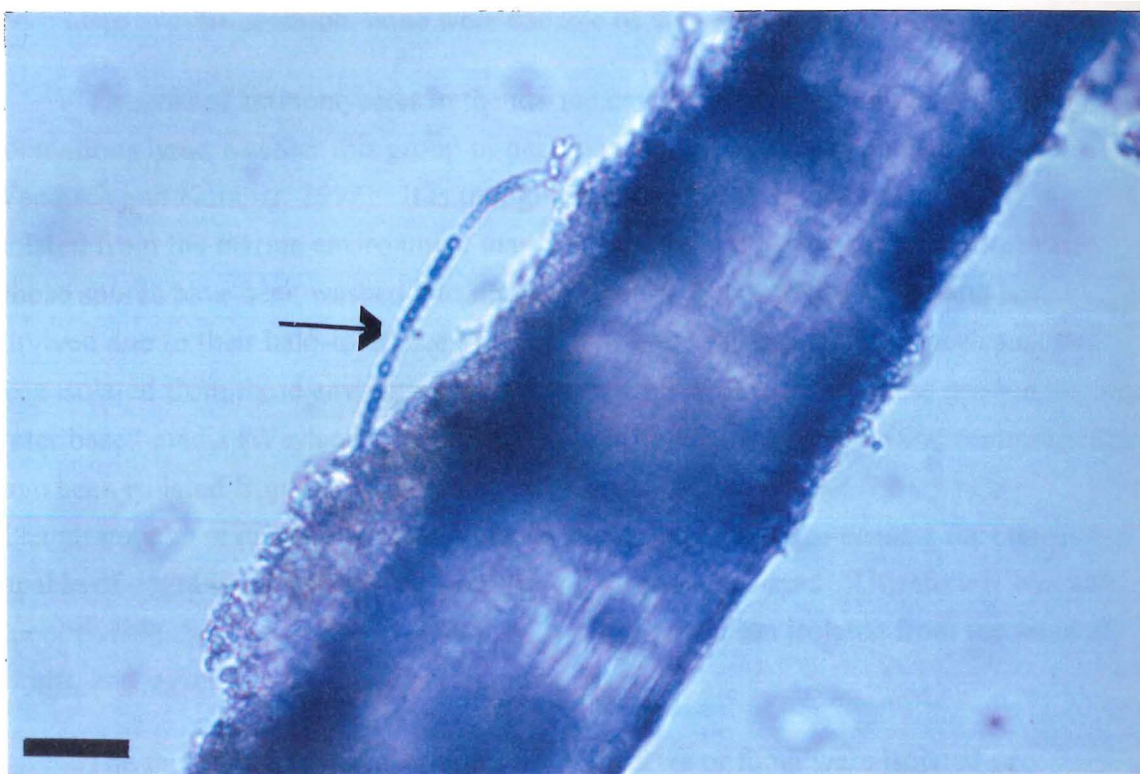
Degradation of the cuticle appears localised, with pitting occurring in random areas of the fibre, and not over the entire length of the fibre. Degradation of the cuticle exposes the keratin containing cortical cells. The degradation of this part of the fibre appears to involve a different process than that seen in the degradation of wool by other keratinolytic bacteria (Figure 25). In microscopic studies involving *S. fradiae*, it has been observed that cortical cells are removed in splinters from the wool fibre by sloughing. This occurs due to the degradation of non-keratinous material in the wool fibre surrounding the cortical cells, releasing these cells in splinters from the body of the fibre (Carter *et al.*, 1987; Brady *et al.*, 1990). No cortical cell splinters were seen associated with, or being removed from the wool fibres in the wool oil boom knops, as was observed in *S. fradiae* degradation. This may be due to the fact that the wool was placed in a harbour environment where water would flow rapidly around it, and this may have washed the cortical splinters off the fibre. However, this does not appear to explain why the same process of wool fibre degradation was seen in laboratory studies, while in *S. fradiae* liquid cultures under similar conditions, cortical cell splinters were observed. It therefore appears that degradation of the cortical cells, occurs in a localised pitting method, within the fibre rather than released from the remaining wool fibre, as occurs in the wool degradation by the keratinolytic bacteria, *Lysobacter* and *S. fradiae*.

Degradation of the wool fibres appear to cause the microbial knops to unravel as the wool fibres begin to degrade and cleave. The strong H<sub>2</sub>S odour that appears after 3 months may have been caused by the presence of sulphate reducing bacteria (SRB), or *Enterobacteriaceae*, present upon the wool boom. *Enterobacteriaceae* have been shown to produce H<sub>2</sub>S in sediments high in organic matter (Nielson, 1980 a,b). If production of H<sub>2</sub>S did occur it would mean that anaerobic conditions had developed within the wool oil boom. The production of H<sub>2</sub>S in wool knops was also seen in static laboratory cultures of wool in sea water, with the growth of *Beggiotoa*-like bacteria observed (Figure 26), which are known to occupy the aerobic/anaerobic interface, and utilise H<sub>2</sub>S (Larkin and Strohl, 1983). It is not known if bacteria that produce H<sub>2</sub>S are utilising the sulphur from the cystine in keratin, or from the surrounding sea water. For SRB's their sulphur source is likely to be the sulphate from sea water.



**Figure 25. A Schematic Diagram Comparing Wool Degradation by Sea Water Microbes and the Keratinolytic Bacteria *S. fradiae* and *Lysobacter*.**

Wool degradation by sea water microbes appears to follow a different mechanism to that of the keratinolytic bacteria *S. fradiae*, and *Lysobacter*. In sea water, the wool fibre is degraded by the degradation of the non-keratinous cuticle. This exposes the keratinous cortical cells. This component of the wool fibre is degraded slowly in a localised pitting action. In *S. fradiae*, and *Lysobacter*, the cuticle is removed, and the inter-cellular cement holding the cortical cells within the wool fibre is degraded, therefore releasing the cells in splinters, from the fibre. Once degradation is completed, the cortical cells remain in the liquid media.



**Figure 26. A Light Micrograph of a *Beggiotoa* like Bacterium on a Wool Fibre.**

*Beggiotoa* like bacteria (arrow) were observed on degraded wool fibres in static wool cultures in sea water. This genus is characterised by filaments containing inorganic sulphur bodies (small spherical bodies) (bar = 10  $\mu$ m).



#### **4.2.2. Microbial Isolation.**

By isolating microbes from the wool oil boom that had been enriched in Lyttleton Harbour, it was hoped to isolate the microbes present within the wool fibre biofilm that are responsible for wool degradation.

##### **4.2.2.1. Fungal and Actinomycete Isolation.**

No actinomycete growth was seen, or isolated from the wool fibres. Fungi were spasmodically isolated from the wool oil boom. Although proteolytic activity amongst these fungi proved common, none were capable of degrading KS.

The role of actinomycetes in the marine environment is not known and it is a contentious issue whether this group of bacteria belong in this environment (Korn-Wendisch and Kutzner, 1992). It is thought that many of the actinomycetes that are isolated from the marine environment may well be artefacts of terrestrial environments whose spores have been washed into marine environments through runoff and have survived due to their halo-tolerance (Grein and Meyers, 1958). Actinomycetes have been isolated from these environments due to the ability of their spores to grow upon sea water based media (Weyland, 1981). Actinomycete populations in marine environments, have been isolated from decaying algae (Chesters *et al.*, 1956), and fishing nets (Chandramohan *et al.*, 1972). These isolates have been shown to possess the enzymes capable of degrading the material, from which they were isolated. Therefore it was not inconceivable that keratinolytic actinomycetes may have been isolated from the wool oil booms, and which were capable of degrading wool.

The fact that no wool degrading actinomycetes or fungi were isolated or observed upon the wool oil boom was interesting as these microbes are considered important degraders of keratin in terrestrial environments, and have been recorded as such many times. The lack of these microorganisms may of course reflect isolation conditions, but if wool degrading species of these microorganisms are lacking from this environment, there must be other keratinolytic microbes present that are capable of filling this niche, and therefore degrade wool.

##### **4.2.2.2. Bacterial Isolates.**

A number of bacterial colonies, and rod-shaped bacteria were seen, through microscopic studies, to be associated with the wool fibres. This indicates that these bacteria may play a role in the degradation of wool in the marine environment, especially when it is considered that the formation of a microbial biofilm upon the wool fibres was an important precursor to degradation.

A number of proteolytic bacteria were isolated from the wool fibre at monthly intervals. The ability of marine bacteria to produce proteases appears common

(Kjelleberg and Hakansson, 1977), this was so in this study. Between 76% and 83% of bacteria screened showed proteolytic activity. This may appear to be a high proportion of the isolated bacteria, but this was to be expected as it has been noted in previous studies that the ability of attached bacteria to produce exoenzymes may be 2 to 20 times higher than free-floating, planktonic bacteria (Chrost, 1987). The percentage of bacteria that were capable of proteolytic activity remained constant, with little variation, during the 8 months of sampling.

Some of the proteolytic bacteria were able to degrade KS. KS degrading bacteria increased as a percentage of the bacterial isolates at each monthly sampling period. This may indicate that bacteria with KS degrading ability were becoming favoured within the microbial biofilm of the wool fibre. This may be due to the presence of proteins in the wool fibre that are capable of being degraded by these bacteria, such as those in the cuticle and cortex, while those bacteria unable to degrade these proteins are being exposed to competition for oxygen, nutrients, and possibly space, in the biofilm (Marshall, 1992).

It was interesting to observe that non proteolytic bacteria were unable to degrade KS. This indicates that the ability to degrade KS may involve the use of a general protease which may also be used in the degradation of wool. During the screening of bacteria for their KS degrading ability, it was noticeable that there was a correlation between the ability to degrade KS and the ability to produce large haloes (up to 5 mm in radius) upon MGA.

The keratinolytic bacteria that were isolated from the wool knops were punitively identified by using a range of taxonomic tests. Two species were constantly isolated during monthly sampling, MBA and MBB.

The fact that only two different types of bacteria were isolated from the wool booms may be indicative of culturing conditions. Possible causes of reduced variation could be due to the use of MA. However, this media was used to isolate bacteria from the wool oil boom, due to its ability to isolate a wide range of marine bacteria, as a general survey of bacterial populations upon the wool boom was initially required. Another possible cause of reduced microbial diversity was the use of a pour-plate technique for isolating bacteria, as heat shock may have had a fatal effect upon the bacteria within the sample. This method was used rather than spread plates, as during preliminary experiments, problems were encountered with spreading bacteria overgrowing the plates.

#### **4.2.3. Tentative Identification of KS Degrading Bacteria.**

A range of tests were used for the presumptive identification of the bacterial isolates. The identification of marine bacteria is very difficult and the phylogeny of these species have not been well studied (Austin, 1988). Marine bacteria are commonly gram negative, motile, rods. This was observed in the isolates MBA and MBB, with both being gram negative, and motile by a single polar flagellum. MBB however, was spiralled in shape, while MBA was a regular rod.

##### **4.2.3.1. Assimilation Tests using Modified API 20 NE Strips.**

API 20 NE strips were successfully employed to determine the metabolic activities of the keratinolytic bacterial isolates. A range of modifications were needed due to differences in culturing conditions that exists between marine and clinical isolates. Most of the modifications involved the use of ASW in the place of 0.85% saline, such as the diluent, to ensure that the osmotic balance within the marine bacterial cells was not upset.

The use of MPRBA rather than the AUX media, which is supplied with the API strips, not only eliminated the problem of marine bacteria not growing in the absence of salt, but the phenol red indicator also provided a useful indicator of bacterial growth. Problems were encountered however, as some positive results returned to negative after a further 24 hours incubation. This may be explained by the utilisation of the acid products, which had previously been produced to give a positive result for the utilisation of the carbohydrate.

The incubation times were increased for the API 20 NE strips from 24 and 48 hours, to 48 and 72 hours due to the slower growth rates of marine bacteria compared to clinical isolates.

Both MBA, and MBB appeared to be limited in their metabolic activities. MBA was able to metabolise gelatin, caprate, and was able to produce oxidase, but variation between strains existed for the metabolism of arginine, urea, esculin, and maltose. MBB was able to metabolise gelatin, p-nitro-phenyl- $\beta$ D-galactosidase, mannitol, and caprate, but variation between strains existed for esculin, arabinose, mannose, and maltose.

Although API 20 NE was useful for obtaining information about the metabolic activities of MBA and MBB, the API scoring system was of no use to identification of the isolates as was found in Breschel and Singleton (1992). This is because the scoring system for identification was established using clinical isolates rather than marine isolates.

#### 4.2.3.2. Fatty Acid Analysis.

The use of fatty acids and related compounds to identify bacteria is achievable due to the large variation in these compounds between bacterial species (Microbial ID Ltd., personal communication). The Microbial ID database contains 60000 analyses of strains gathered from around the world, which have had their fatty acids profiled, and with which an isolate can be compared. However, the database is very limited as far as marine bacteria analysis is concerned (Microbial ID Ltd., personal communication).

This can be seen in the identification of isolates MBA and MBB. MBA was not identified as a species in the database, however it was closely related to *Alteromonas haloplanktis*, although it was not similar to other *Alteromonas* or *Vibrio* species in the database (Table 6). The genera *Alteromonas* fits as a genus for MBA. That is because it is a gram negative rod shaped organism which possesses a single polar flagellum. The genus *Alteromonas* was created as a classification for marine bacteria that are *Pseudomonas*-like but have a lower GC content of DNA (Akagawa-Matsushita *et al.*, 1992) (Table 7). A number of *Alteromonas* species have been isolated that are capable of producing enzymes capable of degrading macro molecules, such as polysaccharides (Akagawa-Matsushita *et al.*, 1992), and proteins (Tsujibo *et al.*, 1993).

Bacterial isolate MBB's fatty acid profile was considered a good match to *Acinetobacter iwoffii*. This classification was unlikely as *Acinetobacter* species are described as short, plump, gram negative rods, which are not flagellated (Towner, 1992). As MBB which is a gram negative, spiralled rod with a single polar flagellum, it is therefore unlikely that isolate MBB belongs to the genus *Acinetobacter*. It is possible that isolate MBB has similar fatty acid composition to this genus, and examples of its genus are not present in the database. MBB was therefore tentatively placed in the genus *Oceanospirillum*, due to the bacterium possessing a spiralled morphology, although this genera is normally characterised by bipolar flagella rather than polar a single flagellum (Austin, 1988).

#### 4.2.4. Confirmation of Wool Biodegradation.

The ability of isolates MBA and MBB to degrade Keratin Azure and wool knops were studied to prove that bacteria were not only capable of degrading modified keratin in the form of KS, but also wool. Like the degradation of the wool oil boom observed *in situ*, and also the ability of the bacteria to degrade wool in pure culture was slow. The process of degradation was similar to that which had been seen in the degradation of the wool oil boom, and also of the wool knops in *in vitro* studies, with the appearance of pits within the wool fibres after 3 months.

<i>Alteromonas</i>	<i>Vibrio</i>
<i>A. arantia</i>	<i>V. aestuarianus</i>
<i>A. haloplanktis</i>	<i>V. alginolyticus</i>
	<i>V. campbellii</i>
	<i>V. carchariae</i>
	<i>V. chlorae</i>
	<i>V. cincinnatiensis</i>
	<i>V. costicola</i>
	<i>V. diazotrophicus</i>
	<i>V. fischerii</i>
	<i>V. fluviialis</i>
	<i>V. furnissii</i>
	<i>V. gazogenes</i>
	<i>V. harveyi</i>
	<i>V. hollisae</i>
	<i>V. logei</i>
	<i>V. marinus</i>
	<i>V. mediterranei</i>
	<i>V. metschnikovii</i>
	<i>V. mimiscus</i>
	<i>V. natriegens</i>
	<i>V. ordalli</i>
	<i>V. parahaemolyticus</i>
	<i>V. tubiashii</i>
	<i>V. vulnificus</i>

**Table 6.** *Alteromonas* and *Vibrio* Species within the Microbial ID. Database.

CHARACTERIASTIC	<i>Alteromonas</i>	<i>Pseudomonas</i>	<i>Alcaligenes</i>	<i>Ocenospirillum</i>	MBA	MBB
<b>Cellular Morphology</b>						
Rods	+	+	+	-	+	-
Spirallae	-	-	-	+	-	+
<b>Flagella Arrangement</b>						
Polar	+	+	-	+	+	+
Peritrichous	-	-	+	-	-	-
<b>GC of DNA (mol %)</b>	38-50	55-64	52-68	42-51	?	?
<b>Gelatinase</b>	V	-	-	V	+	+
<b>Utilisation of</b>						
DL-Malate	V	+	+	V	V	V
D-Sorbitol	V	-	V	-	?	?

+ positive, - negative, V variable, ? unknown.

**Table 7. Comparison of Aerobic Gram Negative Marine Bacteria to MBA and MBB.**

(Adapted from Gauthier and Breittmayer, 1992).



#### 4.3. MECHANISMS FOR WOOL DEGRADATION BY BACTERIA IN THE MARINE ENVIRONMENT.

It appears from the results obtained in this study of wool degradation *in situ*, and *in vitro* that the degradation of wool in marine environments follows an unusual pattern. It appears from microscopic observations, that the non-keratinous component of the wool fibre, such as the scales, cuticle, and intercellular cement are capable of degradation by marine bacteria. This is reflected by the liquid culture studies where stain release from Keratin Azure, and free amino groups release from wool knops, increased, as degradation increased.

The fate of the keratinous component of the wool fibre is still relatively unknown. Under SEM it appeared as though the cortical cells were being degraded, as it was missing from some of the pits. In liquid cultures however, the lack of detectable soluble sulphhydryls indicates that the keratin in the wool has not been attacked, since the disulphide bridges have not been disrupted. This indicates that either the keratin component is persisting within the cultures, or the keratin is being denatured in a way that does not involve the disruption of disulphide bonds.

If the keratinous material of the wool fibre is persisting, it may explain why degradation of the wool fibres was very slow. This may also explain why only proteolytic bacteria were able to degrade KS, as the non-keratinous material is being degraded by a protease which belongs to the bacteria isolated from the wool knops. Alkaline proteases have consistently been isolated from bacteria capable of degrading wool. For example the enzymes termed "keratinases" in *S. fradiae* have been shown to possess the features of an alkaline protease (Moriyama *et al.*, 1974), and have also been shown to be effective in solubilising the protein collagen (Moriyama *et al.*, 1967), the main protein of gelatin (Ward and Courts, 1977). Another bacterium shown to degrade keratin, or feathers solubilised in sodium hydroxide is *Bacillus subtilis* in which the enzyme responsible was also shown to have been an alkaline protease (Takami *et al.*, 1990; Dalev and Neitchev, 1991). This enzyme was also capable of digesting collagen (Dalev and Neitchev, 1991).

Alkaline proteases have been isolated from an *Alteromonas* strain (Tsujibo *et al.*, 1993). Although this enzyme was not examined for its ability to degrade wool, or other keratinous containing materials, it does suggest that such enzymes may be present in species of this genus, and therefore the isolate MBA, and possibly MBB, may be producing this enzyme to degrade the non-keratinous components, releasing the keratinous components of the wool, into the surrounding environment.

#### 4.4. EFFECT OF OIL ON THE BIODEGRADATION OF WOOL.

The effect oil adsorption has on wool degradation was studied to determine whether the action of the marine microbes in sea water, was effected by the presence of oil.

Wool degradation in all cultures was greatly decreased by the adsorption of oil onto the wool fibre. The decrease in the rate of wool degradation was also observed in the mixed cultures of marine microbes. Oil considerably reduced the rate of wool degradation, so much so that the degradation of the wool fibre was not seen under light microscopy, after 8 months, even though it was seen at 3 months in cultures solely of wool. The rate of free amino group release was also shown to be greatly reduced by the presence of oil with the level of release never reaching that of the wool only cultures. Similar results have been observed in *in situ* studies. During Woolspill<sup>TM</sup> trials on Lyttleton Harbour, wool boom degradation was greatly reduced when oil was absorbed onto the wool boom (WRONZ, personal communication).

In the keratinolytic bacteria cultures of *S. fradiae*, and *Lysobacter*, the rate of wool degradation was also decreased by the presence of oil. This was seen in a reduction of free amino group release and also in the reduction of wool fibre damage. Although wool degradation was slowed by the presence of oil, the wool was eventually degraded, and the same level of free amino group release observed in both the cultures containing wool plus oil, and solely wool.

The decrease in wool degradation rates with oil, may be attributed to the toxic effects some compounds within oil, such as polyaromatic hydrocarbons, have upon the bacterial populations capable of wool degradation. For example, components in the oil may prevent the growth of bacterial populations, especially with the oil being in such intimate contact with the wool fibres. Further, it has been shown that oil may inhibit enzyme production and activity. Proteolytic, chitinolytic, and cellulolytic activity have all been shown to be inhibited by oil (Floodgate, 1984). This could be due to the bacteria preferring to degrade hydrocarbons rather than proteins, cellulose, or chitin, as it has been observed that keratinolytic activity is decreased in media high in carbon and nitrogen in *S. fradiae*, and the enzymes responsible for wool degradation are not induced (Winkelmann, 1992).

#### 4.5. EFFECT OF ABIOTIC FACTORS IN SEA WATER UPON WOOL DEGRADATION.

Due to the chemical nature of sea water, such as being high in salts, it was important to understand if these components have any effect in degrading wool, or

weakening it for microbial degradation. A tensile strength tester was used to indicate this. These results indicated that wool strength was not effected by the chemical nature of sea water, as the breaking strength of the woollen fabric remained constant, with little variation, during the sampling period. Woollen fabric was used rather than yarn or wool knops due to its ease of use with the tensile strength tester. An equivalent experiment was designed to take place in the marine environment, however due to the loss of the fabric, through human interference, this was not possible. Wool strength tests have been used before to examine the degradation of wool by bacteria isolated from fleece rot (MacDiarmid and Burrell, 1993), and with microbes that degrade woollen textiles (Lewis, 1975). Both studies observed a significant decrease in the strength of wool as a result of microbial attack.

Sterile controls of the culture experiments looking for free amino group release and soluble sulfhydryl production indicated that sterile sea water had no role in degrading the protein of the wool fibre, or disrupting the disulphide bridges. This indicates that microbes present in enrichment cultures must promote the release of these compounds into liquid media from wool.

## 5. CONCLUSIONS.

Nothing stirs up more emotions in the public mind than pictures of oil washed up onto beaches, and the damage to ecosystems which occurs when large amounts of oil have been spilt. Oil spillages into the marine environment are a fact of life as long as oils are transported by, and used at, sea. It is therefore important that sorbents are developed which are not only efficient at soaking up oil, but also environmentally friendly. That is if they are lost in the environment, they are easily biodegradable, so that they themselves do not become an environmental pollutant. In Woolspill™ an efficient sorbent which uses non-synthetic materials has been produced.

Although the solubilisation of wool by microorganisms, has been extensively studied in terrestrial environments, little is known of the same process in marine environments, and even if such a process exists. Through the results of this study it was found that wool was biodegradable in marine environments.

The potential for wool to be degraded by sea water microflora was examined *in vitro*, so that indicators of wool degradation could be monitored. These indicators were compared to those produced by the keratinolytic strains of *Lysobacter*, and *S. fradiae*. It was found that marine microbes resident in sea water were capable of releasing stain from Keratin Azure, a wool based substrate. This was also seen to occur in the keratinolytic bacterial cultures used as positive controls for wool degradation, although at a much faster rate than that which occurred in mixed cultures of marine microbes. Mixed cultures of marine microbes were also capable of releasing amino groups from wool knops, as was the keratinolytic bacterial controls. This indicated that proteins within the wool were being degraded. The mixed cultures of marine microbes however, were unable to release soluble sulfhydryls from the wool. Indicating that the keratin component of the wool fibre was not being degraded, as soluble sulfhydryls are used as an indicator for the disruption of the disulphide bonds.

The lack of soluble sulfhydryls in the liquid culture, indicates that the wool keratin may not be being degraded, and may be released from the wool fibres through the degradation of the non-keratinous component of the wool fibre by a protease, possibly an alkaline protease, as is thought to occur in *S. fradiae*. Alternatively, the keratin in the wool may be denatured via an alternative method to the disruption of the disulphide bonds, so that the protein can be hydrolysed. If such a mechanism exists it has not been recorded in the literature.

Prior to the isolation of marine microbes from the wool oil boom, a plate assay was developed to rapidly screen isolates for their ability to degrade keratin, using so<sup>l</sup> keratin protein (KS) derived from feathers. This assay was tested using the keratino bacterium *S. fradiae*.

A Woolspill<sup>TM</sup> wool oil boom was placed in Lyttleton Harbour, so that a study of wool biodegradation could take place *in situ*, by isolating wool microbes enriched upon the wool boom, and examining the wool fibres using light, and electron microscopy. Wool degradation, in the marine environment appeared to be reliant on the formation of a biofilm. Once this had been established it was noted using microscopy, that wool damage occurred to the wool fibres. The first components of the wool fibre to be degraded were the non-keratinous cuticle. As degradation continued it was observed that areas of localised pitting began to appear upon the wool surface, with some pits appearing to continue deep into the wool fibre. In these pits, areas of the keratinous cortical cells had been removed, indicating that these cells had possibly been degraded, or released from the wool fibre.

The agents of wool, and keratin degradation in terrestrial environments, appears to involve mainly actinomycete, and fungal components of the microflora. Microscopic observations of wool fibres, and isolation experiments showed that this was not the case in the marine environment. Rod shaped bacteria appeared to be important, as these were consistently observed attached to the wool fibres in areas of damage. Rod shaped bacteria were also isolated from the wool boom, and were demonstrated to be proteolytic, and capable of degrading KS.

Through taxonomic studies of the KS degrading isolates, it was found that two species of gram negative rod shaped bacteria (MBA and MBB), were regularly isolated. Both bacteria were shown to have single polar flagellum. While MBA was characterised by being a regular rod, and MBB by being a spiralled rod. Using a modified API 20 NE strip, for use with marine bacteria, the metabolic activity of these bacteria were elucidated. Identification was also sought for these bacteria from Microbial ID Ltd., where their fatty acid profiles were compared to a database of bacteria. This proved inconclusive in speciating the isolates. However, MBA was placed within the genus *Alteromonas*, a likely classification based on phenotypic tests. The identification of MBB was not conclusive, and it was tentatively placed in the genus *Oceanospirillum* due to its spiralled shape, and marine habitat. Both bacteria were capable of degrading wool in liquid culture.

The effect that abiotic components of sea water have on the rate of wool degradation in sea water appeared to be negligible. It was observed that no additional free amino acids, or soluble sulphhydryls were released from wool knops when they were incubated in sterile sea water. Also the fibre strength of wool when incubated in sterile sea water was not decreased by sea water. This indicates that microorganisms play an important part in the degradation of wool in sea water.

An additional experiment was conducted in liquid culture to observe the effect oil adsorption had upon the degradation of wool. It was found that oil decreased the rate of wool degradation in cultures of the keratinolytic control bacteria of *S. fradiae*, and *Lysobacter*, as well as in mixed cultures of marine microbes. This has implications for the rate of wool degradation in the marine environment when a wool oil boom has been soaked in oil.

Through this study wool has been shown to be biodegradable in the marine environment.

## 6. FUTURE RESEARCH.

Due to the lack of knowledge about the degradation of wool in the marine environment there are unlimited areas for further research in this area. For example the degradation of wool under anaerobic conditions, the effect of oil upon wool degradation, the effect of wool on oil degradation, further characterisation of the bacterial isolates and their enzymes, and the fate of keratin, all need further investigation.

Additional studies need to be conducted upon the keratin component of the wool fibre, to determine whether it was being degraded by marine microorganisms, or whether it was just being released from the fibre. Information upon this could be ascertained by encapsulating a degraded fibre within resin, and cutting transverse sections through pits to ascertain if the keratin within the cortical cells were being attacked. If the keratin is being released from the fibre then the ultimate fate of the keratin needs to be investigated. For instance does keratin degradation take place in marine sediments.

The role of anaerobic conditions, and anaerobic bacteria in degrading wool needs to be determined. Anaerobic conditions are thought to have occurred in the wool oil boom placed in Lyttleton Harbour, due to the production of  $H_2S$ . The utilisation of sulphur in cystine by the bacteria producing  $H_2S$ , needs to be investigated, as this may disrupt the disulphide bonds in keratin, which makes the protein so resistant to microbial enzymes.

It was interesting to note that oil reduces the degradation of wool in sea water. Further experiments need to be conducted upon this effect to establish what is inhibiting degradation, and what the implications are in extending the life of the wool in the booms. It would also be interesting to establish whether wool degrading bacteria are also capable of degrading oil, and whether components of the wool are capable of promoting the degradation of oil, as it is known that sea water is low in some nutrients such as nitrogen which may be supplied by wool during oil degradation. Preliminary experiments have been conducted in this area and it appears oil is degraded when absorbed to wool (Aislabie and McFarlane, 1992).

Further characterisation also needs to be carried out upon the bacteria isolated from the wool oil booms. The bacteria may be further identified by carrying out molecular techniques such as mol percent GC analysis of DNA, and 16S rRNA analysis. Enzymes from these two bacterial isolates also need characterising to determine what function they provide in wool degradation, and whether these enzymes are capable of degrading keratin, or only capable of degrading non-keratinous proteins. If the enzymes are capable of degrading keratin then the mechanism that is used needs to be investigated.



Further investigation also needs to be conducted into the role of bacterial keratin degradation in terrestrial environments, to determine whether species such as *S. fradiae* are truly keratinolytic, or only able to degrade wool due to their action upon the non-keratinous proteins of the wool fibre. *Lysobacter* as a wool degrading bacterium also needs further investigation as a wool degrading bacterium as it has been largely neglected in the literature. Techniques for studying keratinolytic action upon keratin need to be improved due to the use, in many studies, of modified keratin, which may be considerably degraded, with the disruption of the disulphide bonds within the keratin. The structure of such modified keratin substrates need to be investigated to determine whether they contain sufficient disulphide bonds, so that their degradation by microorganisms can be called representative of keratinolytic activity.

All these areas need to be investigated to further elucidate about the degradation of wool, and wool oil booms in the marine environment, and wool degradation in general.

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## APPENDIX

### MEDIA

#### Artificial Sea Water (ASW)

% (w/v)

2.47 NaCL

0.07 KCl

0.63 MgSO<sub>4</sub>·7H<sub>2</sub>O

0.46 MgCl<sub>2</sub>·6H<sub>2</sub>O

0.01 CaCl<sub>2</sub>

In distilled water.

#### Gelatin Marine Agar (GMA)

% (w/v)

8 Gelatin

In Marine Agar 2216

Gelatin was precipitated from the media after incubation by flooding the plates with 80% (w/v) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

#### *Lyso*bacter Basal Salt Media (LBM)

% (w/v)

0.1 K<sub>2</sub>HPO<sub>4</sub>

0.05 MgSO<sub>4</sub>·7H<sub>2</sub>O

0.02 FeCl<sub>2</sub>

0.01 NaCl

pH 8.2

**Lysobacter Culturing Agar (PC Agar)**

% (w/v)

0.25 Yeast Extract

0.5 Tryptone

0.1 Glucose

1.5 Bacteriological Agar

In distilled water.

***Lysobacter* Culturing Broth (PEP)**

% (w/v)

1 Casitone

0.1  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

pH 7.2

In distilled water.

**Marine Actinomycete Isolation Media (M2)**

% (w/v)

1 Starch

0.1 Bovine Casein

1.6 Bacteriological Agar

In 75% (v/v) sterilised sea water, and 25% (v/v) distilled water.

To prevent fungal growth 75  $\mu\text{g/L}$  of cycloheximide was added after autoclaving.

**Marine Fungi Agar (MFA)**

% (w/v)

1      Glucose

0.01    Yeast Extract

1.8     Bacteriological Agar

In aged sea water.

After autoclaving 0.1% (w/v) Streptomycin Sulphate, and 0.1% (w/v) Penicillin G, was added to inhibit bacterial growth.

**Marine Fungi Gelatin Agar (MFGA)**

% (w/v)

8       Gelatin

In MFA.

Gelatin was precipitated from the media after incubation by flooding the plates with 80% (w/v)  $(\text{NH}_4)_2\text{SO}_4$

**Marine Keratinolytic Agar (MKA)**

ASW overlayed with 4 ml of 5% (w/v) KS in 4 ml of SWA

**Marine Phenol Red Base Agar (MPBRA)**

% (w/v)

1       Tryptone

0.5     NaCl



0.00 Phenol Red

18

0.15 Bacteriological Agar

In full strength ASW

### **Sea Water Agar (SWA)**

% (w/v)

2 Bacteriological Agar

In ASW

### ***Streptomyces fradiae* Basal Agar (SFBA)**

% (w/v)

0.15  $K_2HPO_4$

0.0025  $FeSO_4 \cdot 7H_2O$

0.0005  $ZnSO_4 \cdot 7H_2O$

0.0025  $CaCl_2$

2 Bacteriological Agar

In distilled water.

A KS overlay was added by adding 4 ml of a 5% (w/v) KS to 4 ml of SFBA

### ***Streptomyces fradiae* Basal Salt Media (SFBM)**

% (w/v)

0.15  $K_2HPO_4$

0.0025  $MgSO_4 \cdot 7H_2O$

0.0015     $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

0.0005     $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$

0.0025     $\text{CaCl}_2$

In distilled water.